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THE METABOLISM OF HETERAKIS GALLINAE

I. AEROBIC AND ANAEROBIC RESPIRATION: CARBOHYDRATE-SPARING ACTION OF CARBON DIOXIDE[1]

VERA C. GLOCKLIN AND DONALD FAIRBAIRN
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ONE FIGURE

Recent reports dealing with the carbohydrate metabolism of nematode parasites have made clear the fact that there is a considerable variation between species with respect to oxygen requirements, extent and availability of endogenous carbohydrate reserves, nature of the fermentation products, and so on. The subject has been reviewed by Hobson ('48), Bueding ('49a) and von Brand ('50).

With the purpose of undertaking a detailed investigation of an intestinal nematode, *Heterakis gallinae,* a caecal parasite of poultry was selected for study. This species is not pathogenic, although it is a vector for blackhead, an important protozoan disease of turkeys. It does not show absolute specificity for a given host and may infest a number of different species. Individual parasites are small, but are available in reasonable numbers and survive satisfactorily *in vitro* for a few days. In the present communication are described the methods of handling and sampling *H. gallinae,* the general nature of the aerobic and anaerobic respiration, endogenous carbohydrate substrates, fermentation products, and some preliminary observations on the carbohydrate-sparing action of carbon dioxide.

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[1] With financial assistance from the National Research Council of Canada, and Swift and Company.

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METHODS

Poultry culled from commercial flocks at the time that the birds were moved indoors for the fall and winter months provided the best source of material. These naturally infected birds were sacrificed within two to three months, since the infestation with *H. gallinae* decreased considerably during that period of time. A light infestation, averaging 150 parasites per bird, was obtained at other times as follows. Fully embryonated and infective *H. gallinae* eggs were obtained by culture (Swales, '48). Young chickens (5 to 12 weeks old) were fed 1000 of these eggs in poultry mash over a period of 5 days. The mature parasites were available after 5 to 6 weeks.

H. gallinae adults for *in vitro* study were isolated by emptying the caecal contents of freshly killed birds into 1% saline, and transferring the parasites repeatedly to fresh saline until they were clean. This and subsequent operations were carried out at 41°C. The parasites were then divided into the desired number of samples by dealing them serially, one at a time, into beakers of saline. In this way errors due to variations in size, sex, weight and so on were greatly reduced. The samples were ready for use within two or three hours after removal of the parasites from the host. The usual sample was composed of 50 to 125 individuals weighing 15 to 50 mg. Two samples from each experiment were reserved for weighing. Dry weights were less accurate than wet weights, owing to the small amounts involved, and for this reason all results are expressed in terms of wet weight.

Respiratory gas exchange was determined by Warburg's direct method. Gas mixtures other than air were prepared over 25% sodium chloride solution. Because of the high affinity of *H. gallinae* for oxygen at low tensions, nitrogen and carbon dioxide were carefully freed of oxygen by passage over freshly reduced copper oxide (Elliott, '49).

When setting up an experiment each sample of parasites was blotted dry and transferred in one clump by means of a slender, hooked needle into a 7 ml Warburg flask containing

1.0 ml of Krebs-Ringer-phosphate solution, pH 7.3. The desired gas atmosphere was established by repeated evacuation of the system. Shaking the flask at 98 oscillations per minute provided a maximum rate of oxygen consumption which remained constant for at least 4 hours. The parasites moved freely in the suspending medium, with no tendency to clump together as noted for *Nematodirus* spp. by Rogers ('49).

Glycogen and total reducing substances were determined as follows. The sample of parasites to be analyzed was homogenized in 3.0 ml of ice-cold 0.01 N sulfuric acid, following which 0.5 ml portions of the homogenate were mixed with 0.5 ml of 60% potassium hydroxide (for glycogen determination) or 0.5 ml of 10 N sulfuric acid (for total reducing substances determination). Glycogen was isolated by the usual procedure (Good, Kramer, and Somogyi, '33), hydrolyzed, and determined colorimetrically as glucose (Nelson, '44). Total reducing substances were determined after a 30-minute hydrolysis of the homogenate in 5 N sulfuric acid at 100°C. The hydrolyzate was neutralized carefully and diluted to a convenient volume. Unhydrolyzed protein and other interfering substances were then removed by adding to 1.0 ml of the diluted hydrolyzate 3.0 ml of water and 0.5 ml each of 1.8% zinc sulfate solution and 0.1 N sodium hydroxide, and filtering. Reducing substances were determined in 1 ml of the filtrate (Nelson, '44). Fermentable reducing substances were determined in the same way except that to 1 ml of the diluted hydrolyzate was added 3 ml of a 10% suspension of washed baker's yeast instead of 3.0 ml of water. All reducing values are expressed in terms of glucose.

Volatile acids were determined by chromatography on silica (Fairbairn and Harpur, '50, '51a), lactic acid by the method of Barker and Summerson ('41) and pyruvic acid by the method of Lu ('39) and Bueding and Wortis ('40). Catalase was determined in homogenates prepared in ice-cold distilled water by the method of Jolles as described by Sumner and Somers ('43).

RESULTS

H. gallinae survived at 41°C. in Krebs-Ringer phosphate, pH 6.4 to 7.4, for about three days, and for a somewhat shorter time in unbuffered 1% saline. Glucose added to the medium decreased the viability of the parasites, due possibly to the multiplication of acid-forming bacteria in the solution. In an atmosphere of pure nitrogen survival was equally good and no decrease in motility was observed. Aerobically or anaerobically, the saline medium rapidly became acid.

The sampling procedure which has been described was adequate for the requirements of the investigation. Thus, the variation between samples *in a given experiment* was less than 5%, including errors inherent in the methods employed to assess variation. Table 1 summarizes the wet weight and glycogen values obtained in two separate experiments consisting of 4 and 5 samples, respectively.

TABLE 1

Efficiency of sampling technique

EXPERIMENT	NO. OF SAMPLES	WET WEIGHT (MG)		GLYCOGEN (% OF WET WEIGHT)	
		Mean	Extremes	Mean	Extremes
1	4	34.5	34.2–35.0	1.88	1.85–1.91
2	5	43.8	42.6–44.5	2.35	2.30–2.38

Many of the analytical data obtained from many experiments are summarized in table 2. A striking feature of this table is the great variability *between* experiments, compared with the homogeneity of samples in an individual experiment (table 1). Such wide variation from one experiment to another may be caused by a number of factors, among which would be the nutritive and general physical condition of the host, and the age and extent of the infestation. The desirability of obtaining as much information as possible from a given experiment, within the limits of availability of material, is clear.

Values for glycogen and total reducing substances in table 2 are "zero time" values, i.e., those values obtained immediately after completion of the isolation and sampling procedures. Since these substances subsequently disappear rapidly from the parasite tissues, and since two to three hours elapsed from killing of the host to zero time, *in vivo* values must be somewhat greater than those shown in the table. Glycogen comprised from 10 to 90% of the total reducing substances, with the average value of 59%; a large glycogen content was associated with a low total reducing content and *vice versa.* It will be shown that these two fractions decrease in parallel fashion during *in vitro* survival of *H. gallinae,* and for this reason the two determinations were always made. Total reducing values obtained with copper (Nelson, '44) or ferricyanide (Folin and Malmros, '29) reagents were identical and invariably disappeared completely upon fermentation with yeast. In tissue extracts which contained all the reducing

TABLE 2

Variability in different lots of H. gallinae

Dry weight, total reducing substances (as glucose) and glycogen (as glucose) are expressed as per cent of wet weight. $Q^{air}_{O_2}$ (ww) = μl. O_2 consumed per milligram wet weight per hour; $Q^{air}_{CO_2}$ (ww) (and $Q^{N_2}_{CO_2}$ [ww]) = μl. CO_2 produced per milligram wet weight per hour.

DETERMINATION	NO. OF EXPERIMENTS	MEAN	EXTREMES
Wet weight per worm (mg)	43	0.47	0.17– 0.83
Dry weight (% of wet weight)	30	23.3	19.4 –32.2
Total reducing substances	17	4.68	3.47– 6.67
Glycogen	20	2.75	0.45– 4.01
$Q^{air}_{O_2}$ (ww)	21	0.98	0.56– 1.18
$Q^{air}_{CO_2}$ (ww)	20	0.70	0.29– 1.01
R.Q.	20	0.74	0.61– 0.89
$Q^{N_2}_{CO_2}$ (ww)	7	0.39	0.114– 0.58

substances present in the entire homogenate, about 25% of the reducing value was present in the "free" form, that is, did not require the usual preliminary acid hydrolysis. These extracts were negative for hexoses and pentoses by the paper chromatographic method of Horrocks and Manning ('49), and positive for labile organic phosphates (Benson et al., '50). Sufficient material was not available for further investigation, but it is possible that part, and perhaps most, of the non-glycogen reducing substances consists of phosphate esters which vary considerably in concentration from one lot of parasites to another. For present purposes this fraction is considered as a part of the endogenous carbohydrate reserve.

The $Q^{air}_{O_2}$ of *H. gallinae,* although highly variable, is comparable to that of other intestinal nematodes of the same general dimensions (Rogers, '48), and is roughly equivalent to the Q_{O_2} of mammalian muscle. $Q^{air}_{CO_2}$, like $Q^{air}_{O_2}$, was constant for at least 4 hours under the experimental conditions, although at a lower level. Respiratory quotients, consequently, were less than unity. Carbon dioxide was produced anaerobically (table 2, $Q^{N_2}_{CO_2}$) at a somewhat slower rate. For example, during the first 30 minutes of one experiment $Q^{air}_{CO_2}$ was 0.60, $Q^{N_2}_{CO_2}$ was 0.114. In all anaerobic experiments the rate of carbon dioxide production was large enough to suggest the possibility of an active decarboxylation process. On the other hand, *H. gallinae* produced acids both aerobically and anaerobically, and so all or part of the carbon dioxide production could conceivably arise from reaction of these acids with tissue bicarbonate. The bicarbonate reserves were examined, therefore, by measurement of gas production following addition of sulfuric acid from the side arm of the Warburg flask. From 37.3 mg of parasites 5.8 μl (0.26 micromole) of gas were liberated. An identical sample, incubated for 4 hour produced 33.2 μl of gas plus a further 12.1 μl when acid was tipped into the flask. Clearly, the greater part of the gas formed arose from metabolic reactions, and a small retention (6.3 µl) occurred. Values for $Q^{air}_{CO_2}$ and $Q^{N_2}_{CO_2}$ in table 2 were not corrected for retention. Ammonia production, if it occurred, did not interfere with measurement of the carbon dioxide, since two identical samples, one incubated anaerobically with sulfuric acid in the center well of the flask and one without acid, gave the same apparent gas production.

Clearly, *H. gallinae* is capable of utilizing oxygen, although the caecum, its normal environment, must contain a very limited amount of this gas. It was of interest, therefore, to investigate the effect of various oxygen tensions upon its rate of utilization. This could not be done in a single experiment, and the following procedure was adopted. The Q_{O_2} for a given oxygen tension was compared, in a given experiment, with the Q_{O_2} in air and the results expressed in percentage of the $Q^{air}_{O_2}$. Observations were limited to the first 30 minutes of respiration, for at very low tensions the supply of oxygen was limited, and at high tensions the parasites, which were initially very active, were torpid or moribund within two hours. The results (table 3) show clearly that *H. gallinae* utilized available oxygen very efficiently, for the rate at 7.6 mm (1% oxygen) was nearly half the rate in air. At tensions up to and including 152 mm the data for tensions versus Q_{O_2} follow a hyperbolic course, in agreement with the

TABLE 3

Effect of varying oxygen tension upon oxygen consumption

The Q_{O_2} at 152 mm tension is the average of the 5 experiments (see text for explanation)

EXPERIMENT NO.	O_2 TENSION (mm Hg.)	Q_{O_2}	$Q^{air}_{O_2}$	$\frac{100\ Q_{O_2}}{Q^{air}_{O_2}}$
1	7.6	0.33	0.75	43
2	38	0.80	0.98	82
3	76	0.77	0.89	87
	152	...	0.84	100
4	266	1.32	0.74	178
5	380	1.50	0.84	178

results obtained from other intestinal nematodes (Rogers, '49) and many small invertebrates (Tang, '33).

Laser ('44) attributed the toxicity of high oxygen tensions towards *Ascaris lumbricoides* to the accumulation of hydrogen peroxide in the presence of very low (but otherwise unspecified) concentrations of catalase in this species. The catalase activity of *H. gallinae* was determined as follows. A sample of parasites weighing 73 mg was homogenized in ice-cold, glass-distilled water, and divided into two equal parts. One part was boiled immediately to destroy the enzyme, and *k* values (initial first order reaction constant) were determined on both parts. The catalase activity of fresh human blood was determined simultaneously. In the boiled sample the value of *k* per gram wet weight of parasites was 0, and in the unboiled sample was 80; *k* per gram of blood was 610. Since blood is one of the best sources of catalase known, the concentration of this enzyme in *H. gallinae* is not inconsiderable, and the cause of death at high oxygen tensions can scarcely be attributed to lack of catalase without additional supporting evidence.

H. gallinae utilized its endogenous carbohydrate reserves both aerobically and anaerobically. The data for figure 1 were obtained by dividing one lot of 513 worms from 7 birds into 9 samples weighing 35 mg each. Two samples were used for weight determination and one for determination of initial glycogen and total reducing substances. The remaining samples were placed in 3 ml of Ringer-phosphate solution and incubated at 41°C., three in air, and three in nitrogen atmospheres. One aerobic and one anaerobic sample was removed for analysis of glycogen and total reducing substances after 4, 8, and 21 hours. The aerobic and anaerobic glycogen consumption per gram of worm per 21 hours was 28.8 and 32.7 mg, respectively. Clearly there is little or no Pasteur effect apparent in this species, the considerable rate of oxygen consumption (table 2) being superimposed upon a basic aerobic or anaerobic fermentation. Evidence for the existence of a large aerobic fermentation was also gathered from several experiments in which the amount of oxygen consumed was compared with the reducing substances utilized. Thus, in one experiment 22.8 micromoles of glycogen and 24.2 micromoles of total reducing substances (both expressed as glucose) were utilized aerobically per gram of worm in 4 hours. To oxidize this amount of glucose completely would require 137 or 145 micromoles of oxygen, respectively. The measured oxygen consumption was 49.6 micromoles or about 35% of the required amount.

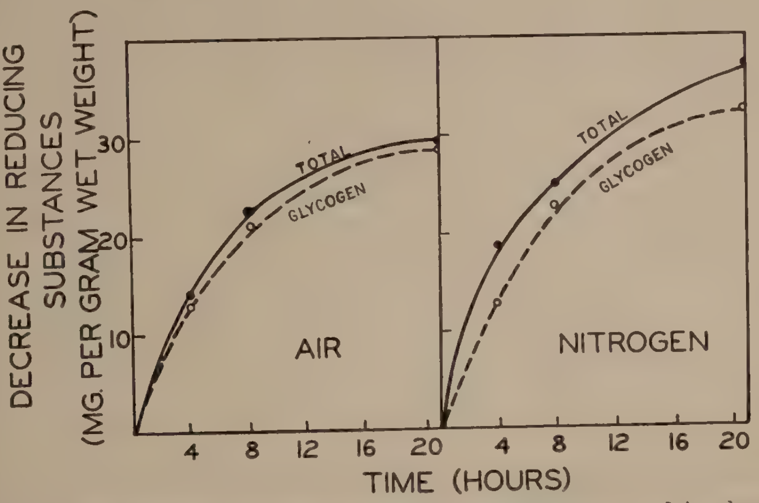

Fig. 1 The decrease in endogenous total reducing substances and in glycogen over a period of 21 hours, in air and in nitrogen. ''Zero time'' values for total reducing substances and for glycogen were 51.0 and 40.3 mg per gram wet weight, respectively.

Fermentation acids were produced both aerobically and anaerobically. A large proportion of these acids (60 to 70%) were volatile. The volatile acids were distilled from the Ringer-phosphate solution and characterized by silica chromatography. The residue from the distillation was made alkaline, and extracted continuously with ether for 24 hours, to remove non-acidic, ether-soluble substances. It was then acidified to congo red paper by addition of sulfuric acid, saturated with sodium chloride, and again extracted continuously with ether. Non-volatile acids in the ether extract were estimated by titration. Table 4 contains the data obtained from two 24-hour experiments, one aerobic and one anaerobic, in which 413 and 380 mg of parasites were incubated in 50 ml of Ringer-phosphate solution in air, and in a Thunberg tube under nitrogen, respectively. Thirty milligrams of penicillin and 50 mg of streptomycin added to each vessel kept the bacterial population at a very low level, as determined by smears made at the end of the experiments. These experiments were carried out on different days and so the usual sampling techniques were not used. The results are in agreement with those obtained from a number of 4-hour experiments, which will be reported in detail elsewhere.

TABLE 4

Aerobic and anaerobic fermentation acids

The results are expressed in micromoles per gram of wet weight per 24 hours

ATMOSPHERE	VOLATILE ACIDS			NON-VOLATILE ACIDS			REDUCING SUBSTANCES UTILIZED	
	Acetic	Propionic	Higher acids	Lactic	Pyruvic	Other	Glycogen	Total
N_2	26	171	5.3	2.4	2.6	58	49	172
Air	63	168	7.6	2.2	1.8	63	76	143

Acetic and propionic acids accounted for 97% of the total volatile acids, the remainder consisting of traces of butyric and higher acids. Analyses for lactic and pyruvic acids by standard colorimetric procedures were positive. Paper chromatography of the non-volatile acids by the method of Lugg and Overell ('48) revealed the presence of lactic and small amounts of two unidentified phosphate esters. This method is relatively insensitive to pyruvic acid. In addition, another non-volatile acid separated from the others and formed a strong spot on the paper. The R_F value of this last acid was somewhat greater than that of any of the common non-volatile acids studied by Lugg and Overell, which suggests that it may be related to the C_5 or C_6 monocarboxylic keto acids recently studied by Liberman, Zaffaroni and Stotz ('51).

Without complete identification of fermentation products it is not possible to calculate material balances between endogenous substrates and end products. Data on the utilization of glycogen and total reducing substances are included in table 4 to emphasize the quantitative similarity between aerobic and anaerobic fermentation in *H. gallinae.*

During the investigation it was observed that the rate of utilization of glycogen and total reducing substances by *H. gallinae* decreased considerably when the carbon dioxide liberated under aerobic or anaerobic conditions ("metabolic" carbon dioxide) was allowed to accumulate in the system. Furthermore, whereas anaerobic carbon dioxide production in phosphate buffer was easily measured manometrically, no net liberation of this gas occurred in bicarbonate-CO_2 buffer. These effects suggested the possibility of carbon dioxide fixation, and a concomitant carbohydrate-sparing effect. Fixation was also suggested by the low respiratory quotients (table 1), for assuming complete combustion of glycogen *and* a simultaneous carbon dioxide production from the aerobic fermentation, the R.Q. would be considerably larger than unity.

TABLE 5

Carbohydrate-sparing action of CO_2

ATMOSPHERE	NO. OF EXPERIMENTS	% REDUCING SUBSTANCES UTILIZED IN 4 HOURS	
		Glycogen	Total
N_2 (CO_2-free)	4	37	44
N_2, metabolic CO_2	3	23	31
N_2, 5% CO_2	3	13	11
Air (CO_2-free)	3	32	33
Air, metabolic CO_2	4	21	20
Air, 5% CO_2	1	17	13

The carbohydrate-sparing effect was studied in more detail in a series of 4-hour experiments. The results (table 5) demonstrated clearly that this phenomenon is extensive and clear-cut in nature, and occurs under aerobic or anaerobic conditions. No differences were observed in viability or activity of parasites in carbon dioxide-free or carbon dioxide-containing atmospheres, so that the possibility of an anaesthetic effect of the gas appears to be improbable. Furthermore, an increase in the gas tension to 10% did not harm the parasites, nor did it have any additional sparing effect on carbohydrates. No conclusion concerning fixation, however, can be drawn from these experiments.

DISCUSSION

The results obtained from the present investigation in general confirm and extend our knowledge concerning the respiration and carbohydrate metabolism of nematodes parasitizing the intestinal tract. Q_{O_2} values in air and in lower oxygen tensions are comparable to those reported by Rogers ('48, '49) and Lazarus ('50) for sheep and rat parasites of the same general size. Very large nematodes such as *Ascaris lumbricoides* and *Ascaridia galli* have a significant but lower rate of oxygen consumption (Laser, '44; Rogers, '48). The fact that *H. gallinae* and certain other parasites appear to survive *in vitro* equally well in nitrogen as in air by no means disproves the necessity for small amounts of oxygen under more natural conditions. Until it is possible to maintain such parasites for an indefinite time apart from the host there is little likelihood of determining with certainty the amounts of oxygen (if any) which are essential.

Although Q_{CO_2} values have been reported for several intestinal parasites (Rogers, '48; Lazarus, '50), aerobically and anaerobically, the liberated carbon dioxide was not shown unequivocally to originate from metabolic processes. However, the improbability that all the carbon dioxide is produced by the reaction between tissue bicarbonates and fermentation acids was emphasized by von Brand ('50), and it is now clear that the carbon dioxide liberated by *H. gallinae* has a metabolic origin. Considerable amounts of this gas are produced anaerobically, presumably from decarboxylation of organic acids, and since the same fermentation acids are produced aerobically (table 4) it is probable that some of the aerobic carbon dioxide production originates from such reactions. At the same time, under aerobic conditions additional carbon dioxide will be produced during the complete oxidation of carbohydrate, and there is in addition the possibility of an extensive fixation (table 5). Clearly, the net production of carbon dioxide by *H. gallinae* may have complicated causes, which are reflected in the calculated respiratory quotient. Possibly the same, or additional, considerations apply to other intestinal species, for respiratory quotient values ranging from about 0.6 to 3.0 have been reported (Lazarus, '50).

Acidification of media by surviving nematodes has been observed commonly, but only recently, and in a few species, has the nature of the volatile fermentation acids been determined with certainty. Among the intestinal parasites *Ascaris lumbricoides* produces mainly C_5 and C_6 acids (Epps, Weiner, and Bueding, '50; Fairbairn and Harpur ,'51b) and *H. gallinae* almost exclusively acetic and propionic acids. A tissue parasite, *Litomosoides carinii,* excretes acetic acid, and the non-volatile lactic acid (Bueding, '49b) which is also formed by *Dracunculus insignis* (Bueding and Oliver-González, '50). Evidently each of these species has a distinctive metabolic pattern. With the exception of lactic, non-volatile acids have not been studied in detail, nor is it known, with the exception of the present investigation, whether they are produced in significant amounts. In view of the large non-volatile acid fraction obtained from *H. gallinae* fermentation it is clear that further study is desirable.

A sparing action of carbon dioxide on endogenous carbohydrate has not been reported previously. The possibility that this effect may be associated with carbon dioxide fixation is supported by observations of Rogers and Lazarus ('49), who found a net disappearance of carbon dioxide from anaerobic bicarbonate medium containing *Nematodirus* spp. or *Ascaridia galli.* In the absence of complete characterization of endogenous substrates and fermentation products this problem can be solved only by the use of isotopic carbon dioxide.

SUMMARY

1. The Q_{O_2} of surviving *Heterakis gallinae* averaged 4.2 (dry weight basis). This intestinal parasite had a high affinity for oxygen, the rate of uptake in 1% oxygen being 43% that observed in air.

2. Q_{CO_3} was invariably less than Q_{O_2}. Carbon dioxide was also produced anaerobically, but at a lower rate than in air. The anaerobically produced carbon dioxide was shown to be of metabolic origin. The parasite tissues contained virtually no bicarbonate reserve.

3. Considerable amounts of endogenous reducing substances were found. These were completely fermentable by yeast and as a rule consisted primarily of glycogen. The non-glycogen reducing substances were not identified with certainty, but consist in part of phosphate esters. Both glycogen and non-glycogen fractions were utilized during survival of the parasites.

4. The disappearance of reducing substances was accompanied by acid production, under aerobic or anaerobic conditions. Little or no evidence of a Pasteur effect was observed. Fermentation acids were about 70% volatile in nature, the volatile fraction consisting almost entirely of acetic and propionic acids. The non-volatile fraction contained a large amount of an unidentified acid which was not, however, identical with any of the common intermediary acids.

5. Carbon dioxide had a pronounced sparing effect upon the utilization of endogenous reducing substances. In an atmosphere containing 5% carbon dioxide, either aerobically or anaerobically, the rate of utilization of these reserves was less than one-half the rate observed in a carbon dioxide-free atmosphere.

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INHIBITION OF PLANT GROWTH BY IRRADIATION

IV. GROWTH ARREST VS. EFFECTS ON MITOTIC ACTIVITY

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FIVE FIGURES

Growth is a complex process, and some of this complexity becomes apparent in the pattern of the modification of growth by irradiation. Previous observations on Mung beans showed that even in a single manifestation of growth, i.e., the growth of the primary root in length, there is evidence of a great diversity of responses to irradiation. However, it was possible to analyze all responses obtained as combinations of a small number of discrete components. These components are the radiobiological expressions of distinct growth mechanisms. (In this paper, "growth" will always signify "increase in length".)

Irradiation can produce any amount of growth inhibition in the roots of Mung beans. However, certain final root lengths occur with much greater frequency than others. For example, mean final root lengths of 6.2 or of 2.2 cm can be readily produced by treating dry seeds with appropriate doses. Yet, the intermediate length of 4 cm is found only in a few individual beans, and never as the mean of a set, even if the doses used are between those which produce roots of 6.2 and of 2.2 cm in length. Also, if growing seedlings are irradiated, their growth may be arrested at any time after irradiation, sometimes even at two or more distinct points in the growth of the root. However, growth arrests are most likely to occur after certain well-defined root lengths have been reached. Even if radiation doses are equally distributed over any given range, certain responses occur much more frequently than others; this preferential occurrence must express properties of the irradiated subject. The spectrum of preferred radiation responses may be considered a partial manifestation of the systems of growth promoting mechanisms (Quastler and Baer, '48, '49a, '50).

It is desirable to correlate the growth promoting mechanisms isolated by radiobiological methods with those identified by methods of longer standing. The present report deals with concomitant observations on mitosis counts and growth (section I). The investigation was made in an attempt to correlate mitotic activity, growth arrest, and growth reserve. It has been found that mitotic activity continues after growth has ceased in normal (section II) and in irradiated plants (sections III, V, and VI), and also during temporary inhibition of growth (section V). Mitotic activity during early development does not add to the radioresistant growth reserve[1] (section III). No correlation was found to exist between growth arrest and the degree of temporary inhibition of mitosis after irradiation (sections V and VI). There might be an increased mitotic activity coincident with increases in the growth rate in normal (section II) and irradiated (section VI) plants.

MATERIAL AND METHODS

Mung beans *(Phaseolus aureus)* of a variety known as "Chinese 793-437" were used as experimental plants. Seeds were soaked overnight, and the seedlings planted in test tubes fitted with collars of gray filter paper and filled with tap water. Their roots were allowed to grow between the tube wall and filter paper. Daily records of their growth before and after irradiation were taken.

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[1] The "growth reserve" or "growth expectancy" of a root, at a given moment, is the difference between its length, at that moment, and the final length reached by untreated control plants raised at the same time. Radiation in doses beyond the threshold causes gradual abolition of the growth reserve; however, one portion of the growth reserve is erased only by doses about one order of magnitude higher than those which are needed to obliterate the remainder of the growth reserve. This portion is called "radioresistant" in contrast to the "radiosensitive" remainder. As in other cases, the radioresistance is only relative. See sections III and IV.

X-irradiations were administered with a superficial therapy machine operating at 100 kV.p. The seedlings were placed on a block of scattering medium, between layers of soaked filter paper. Irradiations were applied uniformly to the entire plant. Factors: target distance 30 cm, 10 mA., no filter, dosage rate of 165 r/min. (including scattered radiation).

All mitosis counts were made on roots cut and fixed at 4 P.M. except for those cut at short intervals after irradiation. The squash method was used in preparing slides for determination and observation of mitotic figures in the roots studied. Good results were obtained with the following technic:

1. Fix in Carnoy's Fluid for at least 24 hours.
2. Place the root tip in a vial containing equal parts of 95% ethanol and concentrated hydrochloric acid for approximately 5 minutes or until the tissue is adequately macerated.
3. Return the root tip to Carnoy's Fluid for two minutes.
4. Place in 70% ethanol for two minutes.
5. Push small segments of the terminal portion of the root tip into a small drop of stain placed on the slide.
6. Allow the stain to react for two or three minutes or until the desired differentiation is reached.
7. Apply a cover slip, invert the slide over a paper towel, and apply direct thumb pressure over the area of the cover slip, being careful not to let the cover slip move.
8. Seal the slide with a mixture of gum-mastic and paraffin.

Two dyes have proven particularly reliable in giving selective staining in our material. An acid hematoxylin stain gives brilliantly differentiated cells in one or two minutes, but is not permanent. If a permanent slide is desired brilliant cresyl blue, although not giving as powerful a stain as hematoxylin, is suitable (Stewart and Schertiger, '49).

The counts were made by one observer who at the time of counting was not informed of the age of the root and the treatment given. In a few cases, counts were done independently by two observers, and a close agreement was found.

NORMAL ROOTS

Among the series of mitotic counts studied were three sets of observations covering the whole period of growth of the root. These sets were taken several months apart. They are fairly representative of the occurrence of mitoses in roots of normal Mung beans, under the existing experimental conditions.

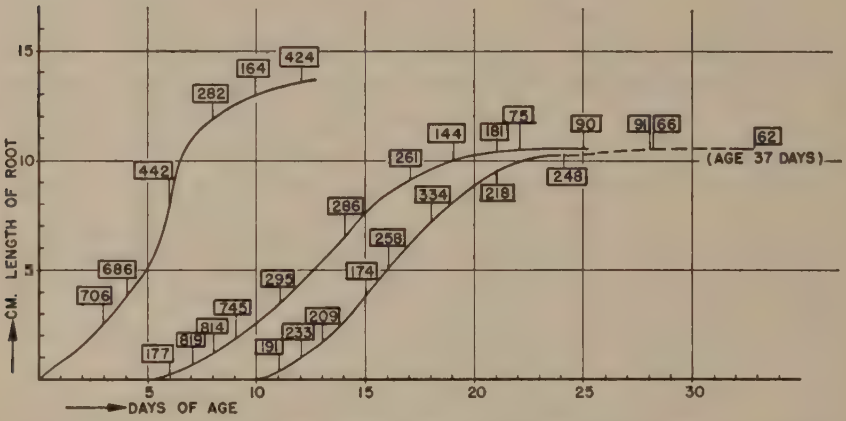

Fig. 1 Normal roots: linear growth and mitotic activity vs. age. The growth cúrves were obtained by averaging the entire set of beans observed at the same time. Individual plants vary slightly from the average. Mitosis counts are entered in boxes; a line connecting the box with the point of the curve indicates the time at which the count was made. Curves a, b, and c represent observations made several months apart.

The three curves of figure 1 show the relation between number of mitoses, growth, and age in normal roots. A substantial number of mitoses was found in the youngest roots examined. With the approach of the period of maximum growth, high mitosis counts around 700 are sometimes found. During the period of maximum growth, the number of division figures in the embryonic zone is around 300. As the growth rate decreases, mitosis counts diminish. Mitotic activity continues for a considerable period of time after the final length has been reached.

The above observations indicate that the limitation of the final length achieved by normal roots is not due to their failure to produce new cells. Therefore, the growth limiting mechanism must affect the process of elongation. Of course, the processes which terminate growth in normal and in irradiated plants are not necessarily identical.

Dry seeds treated with high doses

Seedlings developing from seeds irradiated with 100,000 r respond almost without exception in the following manner: development is severely curtailed, with the epicotyl showing no appreciable development. However, the emerging primary root looks healthy for quite some time. It reaches a final length of about 2.2 cm. This final length of 2.2 cm is a *stable* value of the response, i.e., it occurs unchanged over a wide range of doses. After raying dry seeds with 45,000 r, 50% of the roots reach about 2.2 cm; with 80,000 r, almost all roots reach the same length; with 150,000 r, the 2.2 cm root still predominates; doses around 250,000 r are needed to reduce growth below 2.2 cm. The value of 2.2 cm in seedlings developing from irradiated dry seeds has been designated by the letter "C."[2] Its stability, when compared with changes of dosage, indicates that it expresses a property of the plant. This property may be called the *radioresistant growth reserve.* The value "C" is a measure of the effective amount of radioresistant growth reserve present in the seed.

It is not known what makes a certain portion of the growth reserve (the portion permitting growth up to the value "C") more resistant than the remainder. It was suspected that the answer might be in the process of mitosis. In cytological terms, the supply of embryonic cells which have not yet elongated or differentiated constitutes one kind of "growth reserve." A second kind of growth reserve may be considered to be the number of progeny which the cells of the embryonic zone will be able to produce. It is known that the process of cell elongation is much more radioresistant than the process of cell division (e.g., Forssberg, '34). Thus, theoretically, conditions should exist where the growth of irradiated roots is limited to the amount which can be derived from the elongation of the existing supply of embryonic cells.

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[2] The stable values making up the spectrum of responses were designated by letters of the alphabet; "A" is the normal root length which is stabilized by a threshold effect, "B" the lower limit of mean root lengths in beans which are stunted but completely developed.

The limitation of growth to a final length of 2.2 cm after irradiation with 100,000 r is not due to absence of mitoses. This is graphically shown in figure 2. The mitosis counts are low, but a substantial number of new cells are formed. As in the root tips of normal beans, mitoses keep occurring even after the final length is attained.

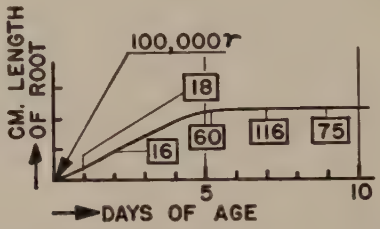

Fig. 2 Mitosis counts and average growth in roots developing from seeds irradiated with 100,000 r. Counts in controls above, in irradiated beans below the curve.

Even doses which suppress any measurable elongation do not completely abolish mitotic divisions. Dry seeds were subjected to doses of 0, 100,000, 200,000 and 300,000 r, and after two days one bean of each set was fixed and studied. The control root had reached a length of 1.2 cm and contained 615 mitotic figures. The bean treated with 100,000 r had grown about the same amount (1.3 cm), and showed a mitosis count of 226. The bean rayed with 200,000 r showed reaction type "E" (Quastler and Baer, '48), a sicklish looking, waxy root of 0.7 cm length; it still contained 93 mitotic figures. The root of the seed rayed with 300,000 r had shown no growth; the mitosis count was 30.

Significance of the early appearance of mitoses in normal roots

The stable value "C," previously mentioned, is found not only in the response spectrum of the dry seeds, but it can be demonstrated in seedlings of any age. However, the dosage range in which "C" is found shifts into lower dosage regions with increasing age. In other words, both the "radiosensitive" and the "radioresistant" portions of the growth reserve become more radiosensitive during development. But, these two portions of the growth reserve remain distinct from one another at all times, and are separable by the irradiation test. Thus, a certain amount "C" is always a distinctive portion of the effective amount of growth reserve, even if the degree of radioresistance and the absolute length connected with "C" change during development.

Until the time the primary root reaches a length of between 1 and 1.5 cm, the stable final length attained by primary roots in the response "C" remains the same. The final length is the sum of linear growth prior to and after irradiation; in the case of a "C" response, it is the sum of actual length at the time of irradiation plus radioresistant growth reserve. The implication is that growth proceeds at the expense of the radioresistant growth reserve. The effective amount of radioresistant growth reserve present in the ungerminated seed is 2.2 cm; it remains the limiting factor in "C" responses until the primary root reaches some length between 1 and 1.5 cm. Yet, as figure 1 shows, a considerable number of new cells have been formed before that time. Therefore, the supply of embryonic cells can be increased without increase of the effective amount of radioresistant growth reserve.

The statement that the amount of radioresistant growth reserve is not identical with the supply of embryonic cells has been strictly proven only for the ungerminated seed. After the intial decrease, the effective amount of radioresistant growth reserve increases again, and reaches about 3 cm during the period of maximum growth rate. We assume that "C"-responses in plants of all ages are homologous. This is not necessarily so. It might be that the substrates of the "radioresistance" of a certain portion of the growth reserve are not the same in the ungerminated seed and in the growing seedling. In this case, the final length would be limited by that substrate which has the higher effective amount: the substrate present in the seed if the irradiation is given to the dry seed or the very young seedling. Therefore, additional studies of mitosis counts and growth have been made on seedlings irradiated during the period of maximum growth.

Inhibition of mitosis by irradiation of growing seedlings

The inhibition of mitoses is one of the best and longest known effects of ionizing radiations. For a given set of conditions (given species, kind of cell, type of radiation, etc.) the degree and duration of mitotic inhibition increases with the dose delivered. High doses can produce complete and permanent blocking of all mitotic activity. Irradiation affects not only the number but also the quality of mitoses; various pathological changes are found in mitoses after irradiation. The Mung bean, with its small and numerous chromosomes, is not suited for a qualitative study of mitoses[3]; however, as the effects of radiation on quality and on quantity of mitoses involve different mechanisms, a study of mitosis counts alone is justified. Doses of 5000 r and of 170 r have been used in 4 day old seedlings to obtain the general relationship between doses and degrees of mitotic inhibition.

Five thousand r administered to 4 day old seedlings almost invariably produces a short temporary inhibition immediately after irradiation, followed by a phase of growth leading to final arrest at the level "C." Figure 3 shows mitosis counts superimposed on an average growth curve:

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[3] It is worth mentioning, though, that no chromosomal aberrations were noticed in the course of this investigation.

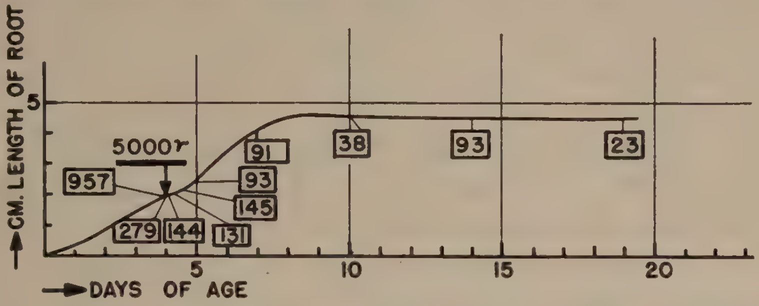

Fig. 3 Mitosis counts and average growth in roots irradiated with 5,000 r. The dose administered produces permanent growth arrest at level "C."

the mitosis count drops to and remains at a low level after irradiation. But, it does not reach zero either shortly after irradiation or for at least 10 days after the final growth arrest. Thus, there must be an appreciable accumulation of unelongated embryonic cells.

Results obtained with 170 r are shown in figure 4. The dose used has no influence on the linear growth, yet, mitoses go

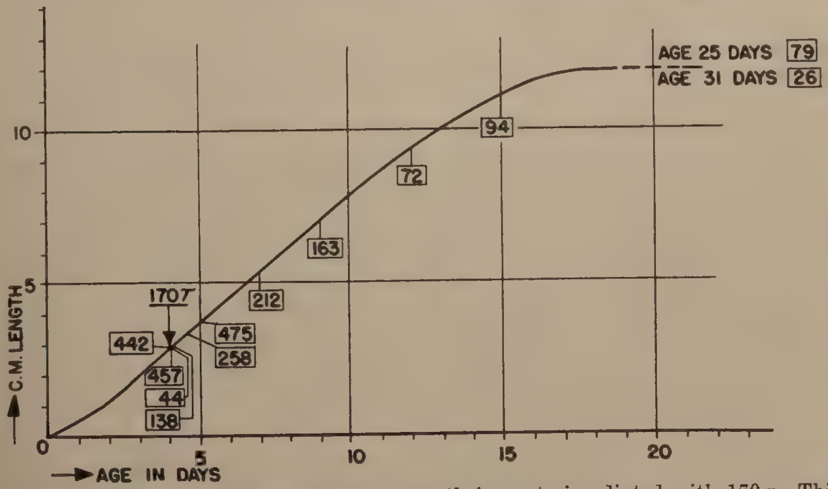

Fig. 4 Mitosis counts and average growth in roots irradiated with 170 r. This dose has no influence on linear growth, but a marked though short affect on mitotic activity.

down to about one-tenth the normal number several hours after irradiation. The mitotic activity returns to normal in about 24 hours, and, from that point on, the subsequent changes in rates of mitosis are as in normal roots. In this case, a short but severe change of mitotic activity had no influence on growth in length.

Mitotic activity during two-phasic responses

Growing seedlings can produce complicated curves of post-irradiation growth, and such responses impose the most stringent conditions on tests of correlation between growth and mitotic activity. A typical response to doses in the neighborhood of 2000 r is as follows: first, there is a temporary inhibition of growth immediately after irradiation; this is followed by a phase of growth leading to level "C"; at this level, growth ceases for a few days, then follows a second phase of growth ending usually at level "B." The sensitivity of seedlings to radiation fluctuates from one experiment to the next, thus, one has to use several doses in the neighborhood of 2000 r in order to obtain a set of plants showing the response described.

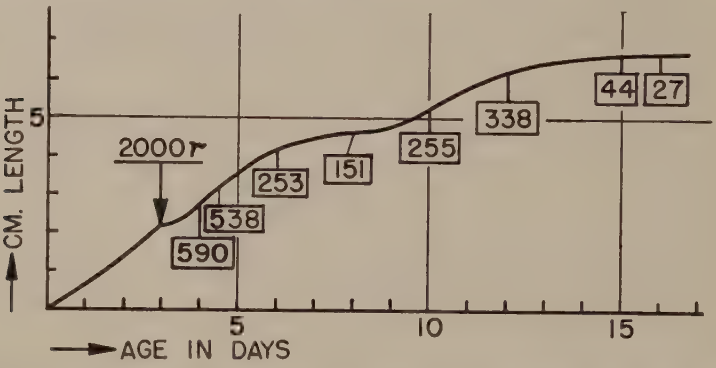

Fig. 5 Mitosis counts and average growth in a two-phasic response, after irradiation with 2000 r.

Three series of mitosis counts were made in the course of two-phasic responses. One of these has been graphed in the usual manner (fig. 5). In this experiment, controls were examined throughout the whole test; the values obtained are entered in figure 1 (left curve). The other two tests will be briefly described. In the first set 4 day old beans, with root lengths of about 2.8 cm, were rayed with 2000 r. Two controls at the time of irradiation had 199 and 370 mitoses. Six days after irradiation one bean, after having stopped growing for two days at level "C," showed 140 mitoses. Two beans were studied after 9 days. One had stopped growing at "C" for 5 days and had 112 mitoses, while the other was actively growing in the second phase and showed 218 mitoses.

Four day old beans, but with an average root length of 2.3 cm, were also rayed with 2000 r in the second set. Three controls at the time of irradiation showed 270, 378, and 396 mitotic figures. Roots after 4 and 5 days, during the slowing down period on reaching level "C," had 98, 76, and 141 mitoses. Six days after irradiation, just after the beans had stopped growing at "C," the tips showed 51 and 53 mitoses. After 8 days and before the beginning of the second phase of growth, 213 and 254 mitoses were found; after 11 to 15 days and during the second phase were 276, 421, 165, 365, 284, 570, and 370 mitoses; after 17 days, at the end of the second phase of growth, 174, 24, 198 and 301 mitotic figures were counted.

The results of the experiments with 2000 r administered to growing seedlings show that the severity of the mitotic inhibition is between that observed after 170 and after 5000 r. The first phase of growth after 2000 r is the same as the only phase of growth after 5000 r, in spite of the pronounced difference in mitotic activity. After 2000 r, the growth arrest at "C" coincides with a mitotic minimum, and the beginning of the second phase of growth with the return of high mitosis counts. This correlation is not necessarily significant; it fails to apply to the final growth arrest which occurs at a time when mitotic activity is still fairly high.

CONCLUSION AND SUMMARY

Cell elongation is notoriously less radiosensitive than cell division, thus, there should exist situations where post-irradiation growth is limited to the amount which can be derived from the elongation of the present supply of embryonic cells. It seemed likely that such a situation is the "C"-reaction where all but the the "radioresistant growth reserve" is abolished by irradiation. The experimental evidence shows that this is not so. Mitoses keep occurring even when growth is reduced to the radioresistant portion of the growth reserve, and, in smaller amounts, even when all measurable linear growth is obliterated. Degrees of mitotic inhibition are very poorly correlated with degrees of inhibition of linear growth; as, continuation of mitotic activity is observed in irradiated and in normal plants long after each evidence of linear growth has ceased. It is certain that the radiation effects on mitosis will have some influence on linear growth; but, such influences cannot be simple and immediate. The radiation-induced inhibition of growth which was observed in bean roots is not directly due to radiation effects on mitosis.

Thus, the radiation effect on growth must affect some mechanism which operates on the cell after it has been formed; it must interfere with the process of elongation. This makes one think of auxin, and of Skoog's ('35) convincing demonstration of radiation effects of auxin. However, Skoog was concerned with irradiations of lesser amount than those employed in this study. There is little doubt that inactivation of auxin is responsible for the immediate temporary inhibition of growth. However, it is unlikely that auxin is the limiting factor in the "C"-response.

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Note: Since this paper went to press, the investigations of Gray and his associates on radiation damage in the broad bean root have yielded a different line of evidence that radiation-induced stunting is not due to inhibition of mitosis. It appears that the critical effect is chromosomal damage, with subsequent cell death after mitosis. See Thoday, J. M. 1951 The effect of ionizing radiations on the broad bean root. Part IX: Chromosome breakage and the lethality of ionizing radiations to the root meristem. Brit. J. Radiol., *24:* 572–576 and 622–628.

TIME OF NUCLEAR, CYTOPLASMIC AND CORTICAL DIVISION OF THE CILIATED PROTOZOAN, TETRAHYMENA GELEII

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INTRODUCTION

Extensive work has been done on the growth rate of *Tetrahymena geleii* (Baker, '42–'43; Browning, '49; Carpenter, '43; Dewey, '44; Elliott, '33, '35, '39; Hall, '33; Hall and Cosgrove, '42; Hetherington, '36; Kidder, '41; Pace and Ireland, '45; Phelps, '35, '36, '46). In summary, the factors which have been found to modify growth rate are nutrition, inorganic ion concentrations, osmotic pressure, oxygen concentration, CO_2 concentration, organic wastes, light, temperature, and the particular strain used. In addition to these factors in the physical and chemical environment of the organisms under study, the history, with respect to exposure to agar and to different temperatures, is significant for many generations (Browning et al., '50). This effect might be called *dauermodifikation.*

An attempt has been made to establish a set of rigidly controlled conditions which is defined as normal, to determine under these conditions the length of time required for the population to double (hereinafter referred to as generation time), and to determine the length of time required for an organism to undergo cytoplasmic cleavage.

It will be shown that the generation time of a culture of Tetrahymena under our experimental conditions increases with an increase in population density, and that this relationship is probably due to decreasing oxygen tension. In addition, it will be shown that the average time of division (''fission time'' — Ormsbee, '42) is a constant up to approximately 150,000 organisms per milliliter, regardless of generation time changes caused by decreasing oxygen tension.

METHODS

Culturing conditions were maintained constant within the limits permitted by available equipment; the following are individual items considered.

Temperature. The temperature was held at 28.28 ± .04°C.

Light. A very low light intensity was maintained and most of the experiments were done with a black, plastic curtain surrounding the constant temperature bath and organisms.

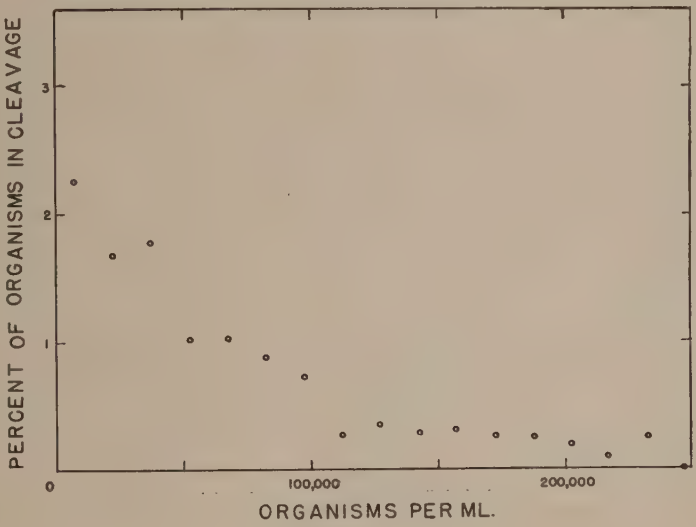
History. The organisms studied were all descendants of a single organism isolated from a contaminated culture December 30, 1949. The culture was *Tetrahymena geleii* (Thomas-Phelps strain) obtained from Dr. Austin Phelps. The controlled stock was maintained in media identical to those in which growth rate measurements were made, and were kept at room temperature which, with rare exception, ranged between 23 and 25°C.

Medium. The medium used is identical in salt content to specifications given by Phelps ('35), using 1% Difco Proteose-Peptone as the nutrient. The pH was adjusted up in every case with sodium hydroxide to pH 6.8 ± .15. Such a large pH range in this region introduces some variation (Wingo and Anderson, '51) but was unavoidable under the circumstances.

Sterilization. An ordinary autoclaving procedure was carried out. The average pH lowering as a result of autoclaving was approximately .1.

Culturing technique. The organisms being studied for growth rate were grown in 50 ml Erlenmeyer flasks containing initially 25 ml of medium. Each flask was fitted with a thick-walled capillary siphon (1 mm I.D.). The siphon was wrapped with gauze covered cotton in such a manner that the flask was snugly plugged. The flasks, after inoculation with a bacteriologists' loop or spiral (Wingo, '50), were fastened in position in the immersion constant temperature bath. With rare exception, 2 ml samples were taken at 24-hour intervals.

Counting procedure. After sampling, organisms were fixed with 5% formaldehyde and were counted immediately to avoid the effect of population decrease due to deterioration (Browning et al., in press, '51). A count of individual cells was made with a Sedgwick-Rafter counting chamber. Each cell was classified according to its gross external morphology (see fig. 1).



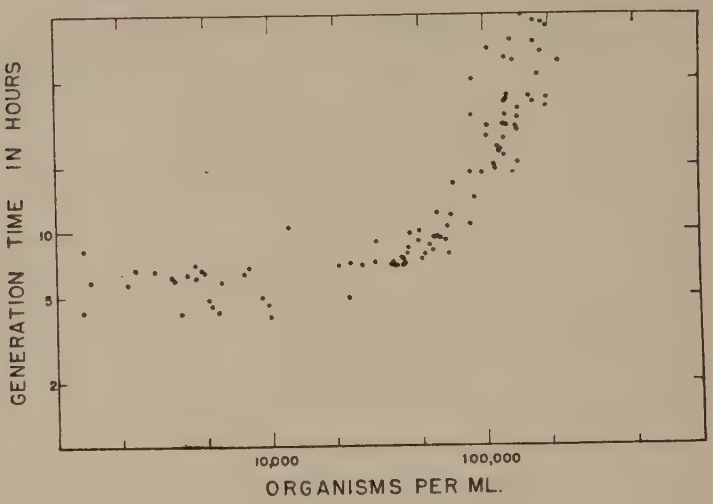
Graph I

DATA AND RESULTS

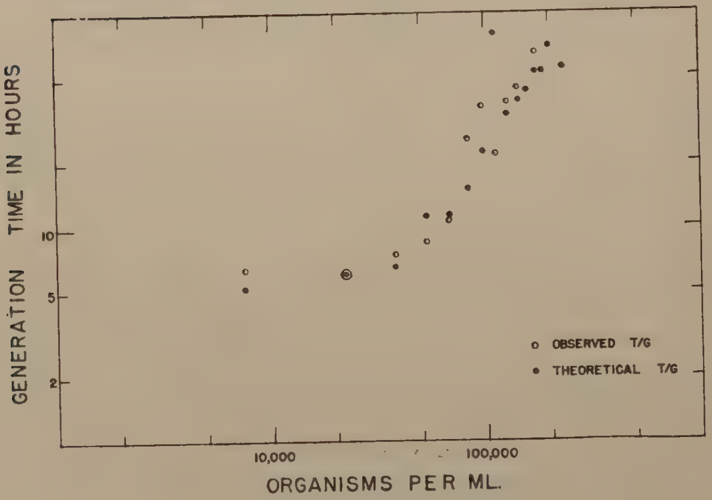
Graph I shows the per cent of the total organisms in division in stages 3 and 4 (see fig. 1 for definition) plotted against population in organisms per milliliter. The per cent is observed to decrease as the population increases.

Graph II shows the logarithm of generation time in hours plotted against the logarithm of the number of organisms per milliliter. Generation times were computed from successive counts in the different experiments.

Graph III shows the logarithm of average generation time of all populations (averaging generation times of all populations between 0–15, 15–30, etc.), whose rate of reproduction was calculated, graphed against the logarithm of the number of organisms per milliliter. Graph III also shows the



Graph II



Graph III

theoretically calculated generation time which may be compared to the observed generation time.

Figure 1 shows schematically the approximate time of cell cleavage in relation to morphological and cytological phenomena as observed by two different methods. Column 1 shows the calculated times based on differential counts made during normal counting procedure. Column 2 shows division time as observed at 950 ×, with a bright contrast phase microscope, on organisms imprisoned in a droplet of normal medium in contact with nutrient agar made of the same medium. This column shows one particular organism which showed slight time variations from — but was generally consistent with — other organisms observed under similar conditions. Column 3 shows nuclear phenomena observed in organisms of the same strain fixed and stained with methyl green. This strain does not appear to differ markedly from that observed by Furgason ('40).

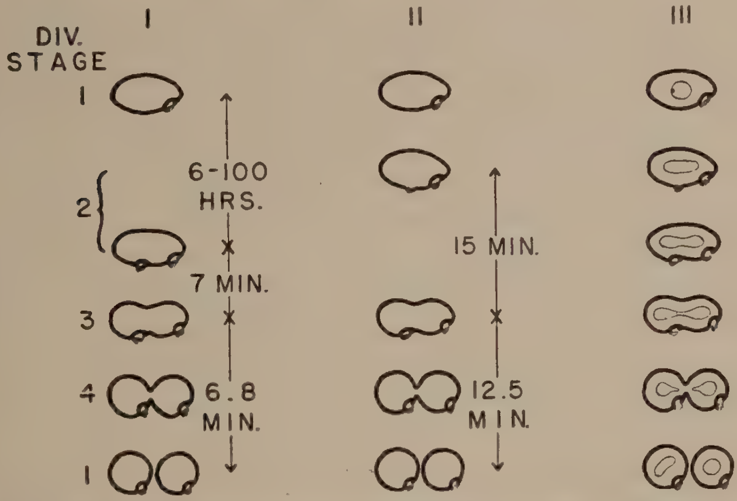


Fig. 1 Schematic representations of classification of stage of division and attendant observed nuclear configurations.
Column I Calculated from growth in normal medium.
Column II Observed in water droplet on nutrient agar.
Column III Nuclear behavior during division.

DISCUSSION

In an attempt to determine the length of time required for Tetrahymena to divide, two approaches were taken. One simple and obvious approach was to watch an organism continuously with the microscope. Several problems accompany this form of observation. In order to find a cell in division at the time that one wishes to make an observation, it is necessary to have a considerable number of organisms from which to choose. Having chosen an organism, one must then continuously watch it until it has divided, carefully distinguishing it at all times from the remainder of the large number. It is essential to have a high enough magnification that the details of division are readily visible, but accompanying the larger magnification a smaller field of vision is available. As a result a mechanical stage must be used continually and with precision, meanwhile, focusing the microscope so that the organism will remain clearly visible at all times. Under these circumstances a relatively small number of observations of the entire division cycle have been completed.

The organisms observed were trapped in a droplet of water on the surface of nutrient agar; consequently, they received very frequent touch stimuli due to collisions with other organisms and stoppage by limits of the droplet. In view of the fact that ciliary motion is modified by touch stimuli, and in all probability the entire metabolic process is somewhat modified, it would be surprising if the division of the nucleus and cytoplasm were temporally independent of environment. Therefore, with respect to column 2, figure 1, it can only be said that the times indicated were observed under the conditions stated, and do not necessarily duplicate the times expected for free swimming organisms in large volumes of medium.

The data obtained in this phase of our study exactly duplicates the finding of Ormsbee ('42) with respect to length of time from initial cytoplasmic constriction to separation of daughter cells. The 12.5 minutes occupied in this division constituted all of the process that Ormsbee could see with 100 × magnification. As will be later explained, these same stages were the only ones that we could reliably detect with 100 × magnification. Since, however, these particular observations of time of division of an organism trapped in a droplet of water were made at 950 × magnification, we were able to observe for 15 minutes preliminary stages of division (see fig. 1, column 2).

A second approach to the determination of division time was made on the basis of aliquot counts. Differential distinction was made between organisms having only a second mouth as an indication of dividing activity (fig. 1, stage 2), those having an equatorial constriction (fig. 1, stage 3), "Figure 8" organisms (fig. 1, stage 4), and "normal resting" cells of the usual pyriform shape (fig. 1, stage 1). It was found that different observers recorded significantly different percentages of organisms in the two mouth stage, other factors being held as nearly constant as possible. Since the observations were made at 100 × magnification with dark field illumination, it was felt that acuity of vision affected the results. This category of organisms, therefore, has been eliminated from our exact computations on the basis of nonduplicability by different observers. An estimated 7 minutes' time from "two-mouth" to " Figure 8" stage is given on the basis of approximate counts. The succeeding classes involving cytoplasmic constriction (pinching in two) and cortical rupture (breaking in two) were easily discernible; the percentages calculated from the numbers observed are summarized in graph I. The assumption on which these calculations were made was that organisms divide in a random manner in any culture containing a large population. This assumption is implicit in all "generation times" calculated on the growth of populations. We found, to our surprise, that at populations around 30,000–70,000 organisms per milliliter the generation time was roughly dependent on the number of samples taken per 24-hour period.

A technique was evolved of taking samples 8 to 24 hours apart and calculating generation times between successive points. Graph II shows the results of these calculations on all of the experiments as listed. There is an obvious relationship between generation time and population density. Some of the variables that account for the change of rate of growth are decreasing oxygen, increasing CO_2, decreasing nutrient available, increasing organic waste, decreasing trace elements. These variables are interdependent to a considerable extent and they had defied analysis up to this time.

Of particular note is the fact that the way the experiment is set up, the amount of oxygen available is limited. Since the oxygen consumption of Tetrahymena is comparatively high (Hall, '38; Baker and Baumberger, '41; Jahn, '36; Ormsbee, '42; and Ormsbee and Fisher, '44) and since oxygen is a growth rate limiting factor (Pace and Ireland, '45) the diffusion rate of oxygen through the small cotton stopper into a volume of liquid with a relatively small surface has become a seriously limiting factor in growth rate of the culture. Lwoff ('32) stated that *Glaucoma piriformis* lives for two or three days but does not multiply under anaerobic conditions. (*G. piriformis* was subsequently identified as *T. geleii* by Furgason, '40.) Phelps ('36) demonstrated a prolonged constant rate of growth in the cultures through which either air or oxygen was bubbled. Other workers have shown a prolonged, relatively constant rate of growth in cultures which were shaken and sampled at regular intervals. These facts led us to suppose, and preliminary tests tend to verify, that oxygen limitation is a primary cause of the characteristic decreasing growth rate shown by graph II.

In our studies two primary phenomena were observed. (1) The per cent of organisms in division decreased with an increase of population density, and (2) the generation time of the culture increased as the population density increased. A relationship appears possible and a working hypothesis to this effect was assumed. If the length of time required for cleavage and breakage into two cells were constant, the above emunerated observations would be accounted for. On this basis, taking the average generation time of all populations between 15,000 and 30,000 as a reference point and computing generations times purely on the basis of the per cent of dividing organisms counted, a series of hypothetical generation times was computed. These values were plotted against population density along with observed generation times (see graph III). An obvious relationship exists between observed and hypothetical values, and the working hypothesis is very close to the truth. It appears that up to populations of approximately 150,000 per milliliter the time required for stages 3 and 4 in the division cycle is independent of the average generation time in the culture. During this phase of culture development, the "mitotic index" is a true criterion of rate of growth. Consistent deviation exists only in very high populations. This deviation is demonstrated in our differential counts of organisms by the diminution and disappearance of constricting organisms (stage 3 of column 1, fig. 1) whereas a small per cent of stage 4 organisms persist for a long period of time. It may be that some trace element that has to do with fragility of the cortex becomes limited in high populations; therefore, the time of breakage increases. Under these circumstances the validity of "mitotic index" to estimate growth rate would depend upon the particular worker's interpretation. If "mitotic index" were determined by counting cells in all stages of division, it would prove to be an incorrect index of reproductive activity in high population densities.

In effect, reproductive rate variation produced by oxygen limitation does not influence time of division; whereas, limitations due to nutrition, ion balance, or other factors, which become effective in dense populations under our experimental conditions, do modify time of division.

The length of time of stages 3 and 4 added together is 6.8 minutes, the time being approximately equally divided between the two stages. This calculation is in reasonable agreement with the observed 12.5 minutes (see fig. 1, column 2) obtained under the adverse and inhibitory conditions previously described.

SUMMARY

Tetrahymena geleii were cultured under oxygen limiting conditions and were observed to have a growth rate characterized by continuously increasing generation time. Division figures were differentially counted and division time was calculated. It was found that division time from the beginning of cleavage to completed fission was a constant (6.8 minutes), and independent of generation time up to approximately three-fifths of the maximum population. Beyond this point division time increased.

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THE OXYGEN EQUILIBRIUM OF HEMERYTHRIN OF ARENICOLA CRISTATA

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ONE FIGURE

Except for the case of the lamprey (Svedberg and Pedersen, '40), the blood hemoglobins of vertebrates are, so far as known, all molecules having a molecular weight in the neighborhood of 67,000 and containing 4 protohemes, which undoubtedly represent the sites of attachment of oxygen. Combination of oxygen with these 4 sites is a complex process and it is certain that there are stabilizing interactions between the sites such that the occupation of one site by an oxygen molecule increases the oxygen affinity of one or more of the remaining sites. This is made clear by the value of $n \cong 2.9$ in the empirical equation

$$Y = \frac{Kp^n}{1 + Kp^n}$$

which gives an approximate description of the equilibrium.[1] In this equation Y is the fractional saturation of the protein with oxygen, K is a constant, and p is the oxygen pressure. Beyond this, there are now grounds for believing that in vertebrate hemoglobins the 4 oxygen combining centres (hemes) occur in pairs with strong interactions between members of the same pair, and lesser interactions between members of different pairs. Moreover there is reason to suppose, due to the symmetry of the curve of Y vs. log p, that the hemes are all inherently alike in regard to their own oxygen affinity and to their mutual relations as represented by the interactions (Allen, Guthe, and Wyman, Jr., '50).

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[1] For a discussion of the significance of n as a measure of the heme interactions see Wyman ('48).

In contrast to the vertebrate hemoglobins the invertebrate hemoglobins, or as they are often called, the erythrocruorins, have very large molecular weights, often running up into the millions, and contain a large number of protohemes. Nothing is at present known about the spatial arrangement or possible interactions of these hemes and most of the data on the oxygen equilibrium leave much to be desired in the way of accuracy. Clearly, in view of the existing picture in regard to vertebrate hemoglobin, it is of great interest to ascertain the precise details of the oxygen equilibrium of some of the large erythrocruorins. A few results, now more than 25 years old and of a rather low order of accuracy by present standards, are available on the oxygen equilibrium of the erythrocruorin of the marine worm Arenicola marina. This is a protein having a molecular weight of nearly 3,000,000 (Svedberg and Pedersen, '40), which, if we assume the iron content to be the same as that of most vertebrate hemoglobins, would imply the presence of approximately 180 hemes in each molecule. These results, on Arenicola hemoglobin, due to Barcroft and Barcroft ('24), indicate that the pigment has a high oxygen affinity (half saturation pressure at 20° and pH 7.3 about 1.8 mm) and that the equilibrium curve is somewhat more sigmoid than that of the mammalian hemoglobins. When recalculated by plotting $\log \frac{Y}{1-Y}$ against $\log p$, the Barcroft data yield a value of n in the neighborhood of 4, it should be pointed out, that a value of $n = 4$ would imply, stabilizing interactions between not less than 4 hemes.

In view of these interesting but tantalizingly meager results, we have reopened the subject by undertaking a study of the erythrocruorin, not to be sure of Arenicola marina, since that species was not readily available to us, but of the closely related species Arenicola cristata, which was obtained from the Marine Biological Laboratory at Woods Hole, Massachusetts.

Although no observations on the molecular weight of the erythrocruorin of this species are available it may reasonably be assumed that it lies in the same range as that of Arenicola marina and that the number of hemes per molecule is much the same in the two cases.

The animals were kept in cool well aerated sea water until required for use. In order to obtain the blood, in which the pigment occurs in solution, not in corpuscles, the animals were pinned down on a sheet of wax in a dissecting tray, the body wall was incised, and all coelomic fluid drained and sponged away as far as might be to prevent possible contamination and dilution of the blood. Then a small tuberculin hypodermic syringe with a conical needle was inserted into the dorsal blood vessel and the blood removed. In a few cases where the blood leaked out of the vessel it was collected with a medicine dropper. In all cases, immediately after removal from the animal the blood was diluted with enough two molar phosphate buffer solution to give a final phosphate concentration of 0.67 molar except in one instance, where the final concentration was 0.50 molar. The resulting pH was between about 7.6 and 7.9. It was felt to be desirable to get the blood to an alkaline pH as rapidly as possible to increase the stability of the preparation. At this stage the diluted blood was centrifuged until clear, at which point it was ready for use. The whole process took less than an hour and was carried out in a cold room at 4°C.

The method of equilibration and determination of percentage saturation of the protein with oxygen as well as of the partial pressure of the oxygen used in these experiments was the same as that previously described by Allen Guthe and Wyman ('50) and need not be gone into here. Since the method required less than 1 cm^3 of the solution to be studied, the yield of blood from a single large worm — 0.6 to 0.8 cm^3 — was sufficient for each experiment. The wavelength employed in the photometric measurements was 710 mμ as in earlier work. The temperature was 20°C.

The results of the experiments are summarized in table 1. In all, 10 experiments were carried out on 7 samples of blood as indicated by the dates in the first column of the table. The oxygen capacity given in column 2 for the sample of August 29 was determined directly by Austin Riggs in a Warburg apparatus. The other oxygen capacities given in the same column were calculated from this as a standard on the basis of the measured optical densities. The final pH values (column 4) were measured directly with a glass electrode.

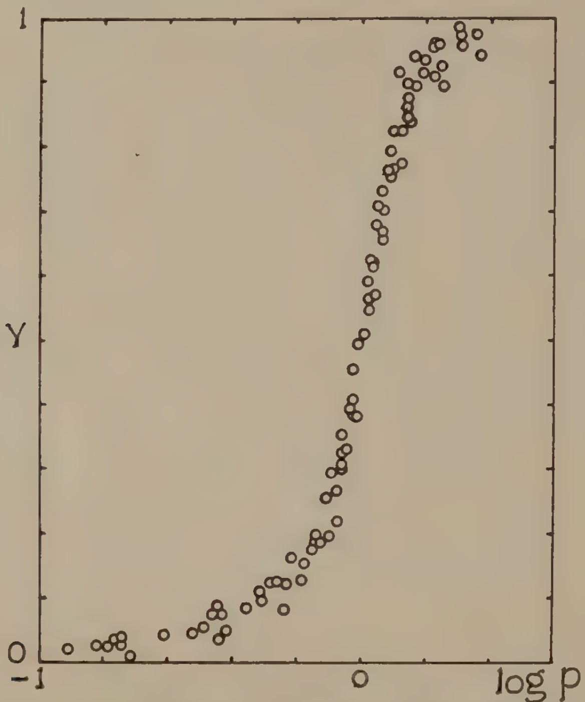

Fig. 1 Combined data on oxygen equilibrium of hemoglobin of Arenicola cristata. Ordinates give fractional saturation of protein with oxygen^{Y}. Abscissae give log $p/p_{1/2}$ where p is oxygen pressure and $p_{1/2}$ is pressure for half saturation.

All the points obtained in the 10 experiments are combined in figure 1. In this figure, following a procedure generally used in studies of vertebrate hemoglobin where the curve of Y vs. log p is invariant in shape for changes of pH, the different sets of data have been brought into coincidence at half saturation by an appropriate shift of each set along the log p axis.

Included in the experiments summarized in table 1, are three additional runs made on the last three days of the investigation to demonstrate the reversibility of the results. The second of the runs on these days was carried out on the residual solution left over from the first. The results, shown in figure 1, are in such complete agreement as to leave no doubt as to the fact that the pigment undergoes no progressive irreversible change during the course of an experiment.

TABLE 1

Oxygen equilibrium of Arenicola cristata at 20°

	DATE	OXYGEN CAPACITY	PHOSPHATE CONCENTRATE	pH	LOG $P_{1/2}$	N
		moles per milliliter				
1	Aug. 25, '51	18×10^{-7}	.67	7.87	.33	6
2	Aug. 26, '51	7.7×10^{-7}	.67	7.87	.24	5.4
3	August 27, '51	6.1×10^{-7}	.67	7.80	.21	5.9
4	Aug. 29, '51	8.6×10^{-7}	.67	7.80	.23	6.7
5	Sept. 1, '51					
	first run	21×10^{-7}	.67	..	.23	5.0
	second run	21×10^{-7}	.67	7.80	.32	5.2
6	Sept. 2, '51,					
	first run	19×10^{-7}	.67	..	.29	5.7
	second run	24×10^{-7}	.67	7.92	.35	5.7
7	Sept. 3, '51,					
	first run	13×10^{-7}	.5	..	.42	5.4
	second run	13×10^{-7}	.5	7.62	.3	5.4

The interesting feature of these results lies in the very large values of n obtained in all the experiments. As we have pointed out, n is an expression of the stabilizing interactions between the oxygen combining centres. A value of $n = 6$ demands the interaction of at least 6 hemes, even if we assume the interaction between them to be infinite (corresponding to the limiting case of a 6th order reaction). Since we may expect the interactions to be less than infinite the very large values of n shown by these data indicate a complicated situation involving the interaction of many hemes. Certainly the protein cannot be regarded as anything like a simple polymer of vertebrate hemoglobin. This extensive interaction might well be accounted for in terms of the hypothesis of configurational changes associated with oxygenation of the protein, which has been put forth elsewhere (Wyman and Allen, '51). It would be of considerable interest to explore the effect of various reagents such as formaldehyde and certain sulfhydryl reagents on these interactions in view of their effects in the case of mammalian hemoglobins containing 4 hemes.

A number of other points warrant brief discussion. In the case of mammalian hemoglobin significant deductions have been drawn from the symmetry of the curve of Y vs. log p. In the case of the present results a good smooth curve drawn through points of figure 1 is not exactly symmetrical, and departs from symmetry in the same way as curves obtained on aged solutions of human hemoglobin. It has been suggested that such an effect is due to changes in the hemoglobin molecule. Whether the lack of symmetry of the present results is to be thought in the same way is of course at present purely a matter of speculation.

As regards the existence and magnitude of a Bohr effect in the case of Arenicola erythrocruorin, the scatter in the observed values of $p_{1/2}$ is too great to make it possible to say much. A comparison of the results at pH 7.62 and those at higher pH suggests however that a Bohr effect not very different from that observed in horse and human hemoglobins may prevail here also. The rather wide scatter in the values of $p_{1/2}$ at essentially the same pH values is somewhat puzzling. It seems not unreasonable however that small molecular changes insufficient to show up in the values of n may have appreciable effects on $p_{1/2}$. The scatter is certainly far beyond the experimental errors.

SUMMARY

The oxygen equilibrium of the erythrocruorin of Arenicola cristata corresponds to a value of n close to 6, and in one case more than 6. This indicates stabilizing interactions between at least 6, and presumably many more, of the large number of hemes present in this molecule. The results do not reveal the exact nature of the Bohr effect in this pigment, but are consistent with the assumption that it is similar to that observed in the case of horse and human hemoglobins.

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THE OXYGEN EQUILIBRIUM OF DUCK HEMOGLOBIN

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ONE FIGURE

According to the data of Wastl and Leiner ('31), which have since been reviewed by Redfield ('33), the oxygen equilibrium of duck hemoglobin is most anomalous. The curve of Y, percentage saturation of protein with oxygen, plotted as a function of p, the oxygen pressure, was reported to consist of two sigmoid portions and to possess two points of inflection. This would make it quite unlike corresponding curves for other vertebrate hemoglobins which possess a single inflection point and are as a first approximation describable by the empirical equation

$$Y = \frac{Kp^n}{1 + Kp^n} \tag{1}$$

in which K and n are constants.

In view of this anomaly, it was decided to reopen the study of the equilibrium, using a new and accurate technique which has recently been described elsewhere (Allen, Guthe and Wyman, '50). At the time of this decision we were unaware of the later work of Christensen and Dill done in 1935. These authors failed entirely to confirm the original results of Wastl and Leiner, and showed instead that duck hemoglobin is not unlike other vertebrate hemoglobins such as those of man and horse, which have been exhaustively studied. Although our results provide a confirmation of the work of Christensen and Dill, nevertheless since they were made with an entirely different method which we believe possesses greater accuracy than any previously employed, and since they bring out one or two features of the equilibrium not attended to by the other workers, it seems worthwhile to publish them in detail.

The following studies on duck hemoglobin were made on solutions prepared from freshly washed cells by essentially the same procedure used in earlier studies on human hemoglobin (Allen, Guthe and Wyman, '50). Approximately 10 cm^3 of blood was taken from the brachial vein of an unanesthetized duck, with citrate as an anticoagulant. The cells were then washed three times with cold 0.9% sodium chloride and were laked by addition of an equal volume of distilled water. In one of the two experiments 0.4 volume of toluene was also added to facilitate laking. After a wait of two hours to allow for hemolysis, the mixture was centrifuged at high speed in a Serval angle centrifuge and the hemoglobin layer pipetted off and recentrifuged. Centrifugation was continued in this way until a clear solution was obtained. This was then buffered with 0.4 molar phosphate of desired pH. One experiment was done at pH 7.10, the other at pH 7.69, each of these being the pH of the final solution studied.

Theoretically it is preferable to plot Y against log p rather than p itself, for reasons which have been discussed elsewhere (Wyman, '48). In the case of those vertebrate hemoglobins which have been studied most carefully, curves obtained in this way prove to be essentially if indeed not exactly invariant in shape for changes of pH and temperature (Allen, Guthe and Wyman, '50; Wyman, '48); that is to say, curves obtained at different values of pH (or temperature) may be made to coincide by a simple translation along the log p axis, the magnitude of which is a reflection of the Bohr effect (or heat of oxygenation). In figure 1 the data of the present two experiments are shown plotted in this way. The points for pH 7.10 have been displaced 0.41 unit in log p to the left to bring them into coincidence with the points for pH 7.69, which were plotted as they stood. It will be seen that the two sets of points are in agreement within the limits of error of the measurements. In this respect duck hemoglobin behaves like other vertebrate hemoglobins. The significant point however in connection with the original problem is that the curve is in general agreement with the results of Christensen and Dill and gives no evidence of the double character (two points of inflection) reported by the earlier workers. It is quite similar in fact to the curves for other vertebrate hemoglobins and has the following properties: (1) It is nearly symmetrical, as is seen from the full line which is exactly symmetrical; although there is a tendency for both the high and low points to lie above the symmetrical curve as in the case of many experiments on other hemoglo-

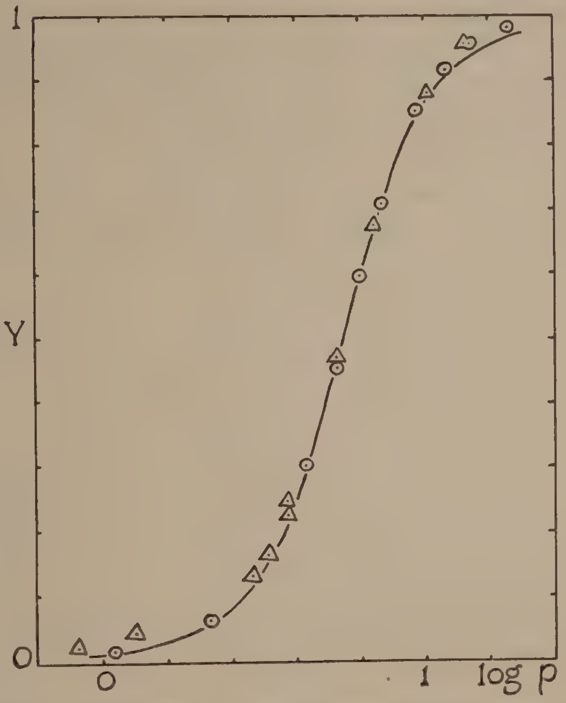

Fig. 1 Oxygen equilibrium of duck Hemoglobin Ordinates give fractional saturation of protein with oxygen (Y). Abscissae give logarithm of oxygen pressure (p) in mm Hg. ⊙ points for pH 7.69. △ points for pH 7.10, translated 0.41 unit to left.

bins.[1] (2) The value of n, defined as the slope of the curve of log $\frac{Y}{1-Y}$ vs. log p at its midpoint ($Y = 1/2$), is 3, as compared with the value 2.9 characteristic of most of the best data on a variety of other hemoglobins such as those of horse and man. This n, which is the same as the n of the approximate equation (1), is a measure of the steepness of the equilibrium curve and a reflection of the interaction of the hemes. It is therefore a fundamental parameter in the description of the oxygen equilibrium. (3) The magnitude of the Bohr effect, represented by the value of $\Delta \log p_{1/2} / \Delta$ pH in the range between pH 7.10 and pH 7.69 is -0.67, which is not far from the value -0.6 characteristic of horse and human hemoglobins in the same range.

SUMMARY

In contrast to reports of earlier investigators, but in agreement with the later work of Christensen and Dill, the oxygen equilibrium of duck hemoglobin is found to be very similar to that of other vertebrate hemoglobins. The curves of percentage saturation vs. log oxygen pressure are found to be nearly if not quite symmetrical and to be invariant in shape for changes in pH. They are of a steepness corresponding to n (equation 1) $= 3$. In the range from pH 7.10 to 7.69 the Bohr effect, expressed as $\Delta \log p_{1/2} / \Delta$ pH, is -0.67 as compared with the value -0.6 characteristic of horse hemoglobin.

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[1] For a discussion of symmetry see Allen, Guthe and Wyman ('50).

HISTOCHEMICAL STUDIES OF ALKALINE PHOSPHATASES IN THE TISSUES OF THE RAT USING FROZEN SECTIONS[1]

II. SUBSTRATE SPECIFICITY OF ENZYMES HYDROLYZING ADENOSINE-TRIPHOSPHATE, MUSCLE- AND YEAST-ADENYLIC ACIDS, AND CREATINE-PHOSPHATE AT HIGH PH; THE HISTOCHEMICAL DEMONSTRATION OF MYOSIN ATP-ASE

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TWENTY-TWO FIGURES

INTRODUCTION

In a previous paper (Davies and Friedenwald, '50) we have shown that is is possible to differentiate histochemically in fresh frozen tissue sections substrate specific enzymes hydrolyzing a number of different phosphate esters. Under the conditions of our experiments the presumed non-specific alkaline phosphomonoesterase was absent or inactive. Differentiation of the substrate specific phosphatases was established on the basis of one or more of the following criteria: differences in anatomical location of activity for different substrates, differences in response to various activators or inhibitors, differences in diffusibility of the enzymes or of some cofactor required for the enzymatic hydrolysis of different substrates. The present study was undertaken to determine whether the methods previously developed could be extended to the demonstration of substrate specific enzymes for adenylic acid, adenosine-triphosphate, and creatine-phosphate.

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[1] This work was supported by a research grant from the National Cancer Institute of the National Institute of Health, Public Health Service.

[2] Fellow in Ophthalmology.

As already pointed out in our previous publication (Davies and Friedenwald, '50), numerous workers using paraffin embedded tissue were unable to distinguish histochemically substrate specific enzymes hydrolyzing various phosphate esters (Gomori, '41, '49; Kabat and Furth, '41; Bourne, '43; Danielli, '46, etc.). More recently Newman, Feigin, Wolf and Kabat ('50) demonstrated the presence of a 5-nucleotidase (Reis, '34; Gulland and Jackson, '38) in dehydrated, paraffin-embedded tissue sections. The localization of ATP-ase was attempted by Glick and Fischer ('45a, '45b, '46), but the substrate specificity of the enzyme demonstrated by these authors has been open to doubt (Moog and Steinbach, '46; Glick, '46).

The tissues and substrates used in the present study are listed in table I. In comparing the results of our present study with those that we have previously reported, it may be stated at the outset, that the pattern of organ and tissue distribution of enzyme activity for each of the presently used substrates and the response of these activities to various activators and inhibitors differ significantly from the various patterns found for the polyglycol phosphates and aryl phosphate employed in our previous study. All of these patterns differ from those found with nucleic acids as substrates (Friedenwald and Crowell, '49; Krugelis, '46). In the present study we have not employed diffusion experiments such as were described in our previous report, since no striking differences in the diffusibility of the enzyme systems were noted in respect to the substrates now being considered. On the other hand, significant differences were found in response to changes in the pH of the incubating solution within the alkaline range.

At pH 8.9–9.0, which was most satisfactory for the substrates previously studied, adenosine-triphosphate was only feebly and irregularly attacked, whereas at pH 9.9–10.0 intense, sharply localized and widely disseminated activity for this substrate was found. With this same substrate at pH 8.25–8.3 intense activity was found. In voluntary muscle and heart muscle the ATP-ase active at this lower pH was strongly activated by glutathione and inhibited by sulfhydryl binders. This activity at the lower pH in muscle is, therefore, attributable to myosin ATP-ase, while that at the higher pH must be due to a different alkaline ATP-ase or pyrophosphatase.

TABLE 1

Tissues and substrates studied

RAT TISSUES STUDIED	SUBSTRATES	SOURCES
Bladder (urinary)	Adenosine triphosphate = ATP (Ba- and Na-salts)	Armour Laboratories
Brain (cortex)		
Colon		
Duodenum		
Heart	Adenosine-5-phosphoric acid = A-5-P	Schwarz Laboratories
Kidney		
Liver		
Lung	Adenosine-3-phosphoric acid = A-3-P	Nutritional Biochemicals Corporation
Ovary and Fallopian tube		
Prostate		
Rectum	Creatine-phosphate = Cr-P (Ca-salt)	Bios Laboratories, Inc.
Seminal vesicle and coagulating gland		
Skin (sole)		
Skin (tail)		
Stomach (pylorus and cardia)		
Striated muscle		
Uterus		

All substrates were employed at the same molarity (0.005 M final) after conversion to the sodium or potassium salt.

Similarly at pH 8.9–9.0 the tissue sections (in the absence of added magnesium) showed no activity for creatine-phosphate, but widely disseminated activity was found if the pH was raised to 9.9–10.0, while at pH 8.9–9.0 in the presence of magnesium a different activity pattern was revealed.

The inhibitors and activators employed were chosen according to observations recorded in the general chemical literature in this field (Banga and Szent-Gyorgyi, '43; Laki, '43; Binkley, Ward and Hoagland, '44; Potter, '44; Barron and Singer, '45; Engelhardt, '46; Szent-Gyorgyi, '47; Libet, '47; Bailey and Perry, '47; Banga, '47; Gordon, '49; Roche, Van Thoai and Bernard, '49, etc.).

The difficulties encountered in using frozen tissue sections instead of dehydrated, paraffin-embedded material for the Gomori ('39)-Takamatsu ('39) technique have already been described (Davies and Friedenwald, '50). All of the improvements on this technique which were worked out previously (Friedenwald and Becker, '48; Davies and Friedenwald, '50), i.e., saturation of the incubation mixture with the final product and high salt concentration, were again employed in this study.

METHODS

White male and female Wistar rats[3] from Carworth Farms, weighing 140–180 gm were used throughout this study. The animals were killed by decapitation, the organs to be examined removed as quickly as possible and immediately frozen on dry ice. Tissue pieces were frozen to the stage of a freezing microtome with half-saturated sodium acetate solution, made up with glass distilled water. Sections 20 to 30 μ thick were cut and dropped directly from the knife into the incubation mixtures described in table 2. These incubation mixtures were buffered by the presence of 0.025 M glycine and also in part by the very high concentration of acetate present. Pilot measurements indicated that the drift toward lower pH during incubation due to liberation of inorganic phosphate amounted to less than 0.2 pH units. The high concentration of acetate was introduced into the substrate solution in order to diminish enzyme diffusion and autolysis. The utility of this procedure is discussed in our previous paper.

Since glycine dissolves slowly in the presence of high salt concentrations, two stock buffer solutions were made up as follows: For stock buffer no. 1 the glycine and potassium chloride were weighed out and dissolved in as little glass distilled water as possible. Then a saturated solution of sodium acetate in glass distilled water was added; but enough space was left to adjust the pH of the buffer with 1 N KOH in glass distilled water and to leave room for the addition of sodium acetate crystals to render the buffer fully saturated when made up to volume. The pH was brought to 9.35–9.4 with KOH and checked again with a glass electrode system when the buffer was completed. The same procedure was used for stock buffer no. 2 of pH 7.45, but the adjustment of the pH in this case was made with 1 N HCl in glass distilled water. Using one or the other of these buffer solutions the final pH adjustment was made by adding KOH to the incubation mixture (see table 2). The final pH for ATP and creatine-phosphate adjusted with KOH, using stock buffer no. 1, was found to be 9.95–10.00. For muscle- and yeast-adenylic acid as substrates with the same buffer the pH was 9.90–9.95; for creatine-phosphate and Mg^{++} it was 8.90–8.95. When ATP was used as substrate with stock buffer no. 2 the final pH was established at 8.25–8.30. All measurements of hydrogen ion concentration were performed with a glass electrode at 25°C. At incubation temperatures of 37°C. the pH would be slightly lower.

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[3] The animals were fed on Purina Laboratory Chow supplemented once weekly with 1 ml of cod liver oil and 1 ml of wheatgerm oil for each animal.

TABLE 2

Incubation mixture [1]

Glycine buffer,[2] pH 9.35–9.4 in saturated sodium acetate (CH_2NH_2COOH 0.1 M, KCl 0.4 M)	2.0 ml
Saturated CH_3COONa	2.0
$CaCl_2$ (0.36 M)[3]	0.6
KOH (1.0 M)[4,5]	0.1
Substrate (0.04 M)	1.0
H_2O distilled [6]	2.3
Saturated Na_3PO_4 [7]	1 drop
Filtered after 1 hour at incubation temperature (37–38°C.).	

[1] All water employed in the ATP and Cr-P experiments was glass distilled.

[2] For the demonstration of the myosin ATP-ase the buffer was employed at a pH of 7.45.

[3] For myosin ATP-ase the amount of $CaCl_2$ (0.36 M) was increased to 0.8 ml.

[4] For the demonstration of a second enzyme attacking creatine-phosphate the KOH was replaced by 0.1 ml $MgCl_2$ (0.36 M).

[5] For myosin ATP-ase 0.2 ml of 0.1 M KOH was used.

[6] For myosin ATP-ase the amount of glass distilled water added was decreased to 2.0 ml.

[7] For myosin ATP-ase 3 drops of saturated Na_3PO_4 were added.

The ATP and creatine-phosphate substrate solutions were made up fresh every third day. They were adjusted to a slightly alkaline pH (9.0–9.5), frozen immediately and kept frozen until used. Both adenylic acid substrates were made once a week and stored frozen at neutral pH.

For the inhibition experiments the tissue sections were exposed for 30 minutes to the inhibitor-mixture described in table 3. They were then transferred to the incubation mixture of table 2 which now contained the same concentration of inhibitor as employed in the inhibitor-mixture, replacing the appropriate amount of distilled water by inhibitor solution.

Owing to the photo-sensitivity of p-chloromercuri benzoate[4] this inhibitor was employed in the dark.

In the activator experiments an appropriate amount of activator dissolved in glass distilled water was incorporated in the incubation mixture of table 2, replacing a part of the distilled water. The glutathione[5] was neutralized with 1 N KOH in glass distilled water and made up fresh for each experiment. Since no special care was taken to exclude air from the experiments, we cannot state to what extent the glutathione was in the oxidized state.

TABLE 3

Inhibitor-mixture[1]

Saturated CH_3COONa	4.0 ml
Water solution of inhibitor (appropriate concentration)	1.0
Distilled water	3.0

[1] Glass distilled water was used for all ingredients of this mixture.

For each experiment about 20 to 25 tissue sections were used. In all types of experiments at the termination of the desired incubation period, the sections were washed with 75% ethanol containing 1% calcium chloride before being mounted on glass slides filmed with albumen. By this procedure the excess sodium acetate was removed without the loss of calcium phosphate deposit. The slides were then dried by careful heating. The calcium phosphate deposited in the tissue by enzymatic action was replaced by cobalt sulfide to make the deposit visible, as described by Gomori ('39), and modified by Davies and Friedenwald ('50).

All experiments were controlled in two ways. One control was run by using an incubation mixture in which the substrate was omitted, and the second control in which, after heat destruction of the enzyme, the tissue sections were exposed to the incubation mixture containing substrate. The heat destruction was performed by boiling thin, hand-cut tissue slices in distilled water for 30 minutes. Subsequently the tissues were sectioned on the freezing microtome and treated as previously described. Both types of controls showed hardly any deposit, i.e., darkening of the tissue sections even after 140 hours' incubation.

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[4] The authors are indebted to Dr. L. Hellerman for the supply of these inhibitors.

[5] Glutathione was the commercial product sold by Nutritional Biochemicals Corp., Cleveland, Ohio.

Differentiation by morphologic pattern. Of the 4 substrates studied at pH 9.9, two, namely adenosine-triphosphate (ATP) and muscle-adenylic acid (A-5-P), exhibited a very similar pattern in all tissues. However, owing to considerable differences in incubation time and slight differences in the resulting histological pattern, they will be described separately. Since it was found that creatine-phosphate (Cr-P) is split by one enzyme in the presence of $MgCl_2$ (4.5×10^{-3} M final concentration) at pH 8.9 and by another in the absence of this activator, at pH 9.9, the activity patterns in the presence and absence of Mg ion will be described. ATP was also employed as substrate at pH 8.25 yielding a different activity pattern from that obtained at pH 9.9. Yeast-adenylic acid (A-3-P) was used at pH 9.9, and the enzymatic activity for this substrate will also be described.

Description of enzyme localization in the tissues and organs of the rat. The histological descriptions which follow show the activity patterns which we have found in numerous experiments in various tissues and organs of the rat. In the case of striated muscle, heart muscle and kidney the incubation times for ATP as substrate at pH 8.25 are compared in the presence and absence of glutathione.

Brain (cortex). After one hour's incubation with ATP as substrate at pH 9.9, blood vessels of all sizes appear clearly stained and the brain tissue itself shows a diffuse homogeneous darkening. When incubating for longer periods the cytoplasm of some neurons appears diffusely darkened, and in some of these, the cell membrane and nucleus show distinct blackening. With these longer incubation periods glial cells and their nuclei show a heavy deposit.

With ATP as substrate at pH 8.25 the nuclei of large neurons and glial cells are faintly active after two hours' incubation. After 22 hours' incubation glial cell nuclei show a heavy deposit in the nuclear membrane and chromosomal detritus. A clear zone remains unstained in the periphery of the nucleus. The nuclei of protoplasmic astrocytes and some of the processes of these cells are very dark, while their cytoplasm stains lighter. Blood vessels of all sizes show activity, but the activity is less pronounced than at pH 9.9.

The enzyme attacking A-5-P yields the same picture in 6 hours' incubation as that obtained with ATP as substrate at pH 9.9 in 3 hours' incubation time.

With A-3-P as substrate the glial cell nuclei show distinct activity in 24 hours' incubation with the cytoplasm being diffusely darkened.

Capillaries and larger blood vessels show strong activity. The nuclei of protoplasmic astrocytes show strong activity, but their processes and cytoplasm are less active, being only faintly darkened. Occasional nerve axons show some darkening. In 30 hours' incubation the neuronal cell membrane is blackened.

With Cr-P at pH 9.9 in 48 hours a slight darkening of protoplasmic astrocytes and their processes can be observed. Other glial cells also appear faintly positive. The medium and small blood vessels are active with this substrate. Occasional neurons show diffuse darkening, especially their cell membranes.

With Cr-P at pH 8.9 in the presence of Mg^{++}, a distinct capillary stain can be observed in 24 hours' incubation time. The nuclei of protoplasmic astrocytes appear darkened with their cytoplasm and processes showing faint activity. The nuclei of other glial cells are very dark with only a faint darkening of the cytoplasm. The cytoplasm of many neurons is slightly active but their cell membranes and nuclei are usually clearly stained.

Lung. With ATP as substrate at pH 9.9 in 5 hours' incubation, the nuclei of the smooth muscle of the bronchi show strong enzymatic activity with the muscle fibers diffusely darkened. Elastic fibers of the lamina propria and the basement membranes are strongly active. The same muscular activity can be observed in the bronchioles, although the stain is not as dark in this location. The columnar epithelium lining the bronchioles shows intense darkening of the basal nuclei with the cytoplasm being only slightly active. In large blood vessels the muscular media shows strong activity, the nuclei being much darker than the fibers. The endothelial cell nuclei also show strong activity. Occasional blackened flat nuclei or a blackened thickening which may represent the walls of the alveolar sacs can be observed. Larger cells, probably phagocytes, in the parenchyma of the lung stain with dark granular masses visible in their cytoplasm. Numerous nuclei and whole cells in the interstitial tissue and the parenchyma show intense activity.

The enzyme attacking ATP at pH 8.25 is less active than at pH 9.9. After 22 hours' incubation the distribution is the same as that at pH 9.9 in 5 hours, except that the nuclei and cells of the interstitial tissue and parenchyma seem more distinct.

A-5-P after 24 hours' incubation yields an almost identical picture with ATP at pH 9.9 in shorter incubation periods. The elastic tissue, however, shows more pronounced activity with A-5-P.

The enzyme attacking A-3-P shows nuclear activity throughout the parenchyma of the lung after 24 hours' incubation. Muscular nuclei and fibers appear darkened, and the bronchial epithelium shows activity throughout the cell. The capillary endothelium as well as the endothelium of larger vessels is strongly active, the nuclei showing the most intense stain.

With Cr-P at pH 9.9 after 48 hours' incubation, the bronchial epithelium shows a heavy deposit with nuclei being occasionally discernible. Elastic fibers stain strongly and the smooth muscle of bronchi and bronchioles is darkened. Occasional cell nuclei in the parenchyma show distinct activity although the entire parenchyma appears diffusely darkened. The media of blood vessels is stained.

With Cr-P and Mg^{++} at pH 8.9 entire cells and some nuclei stain throughout the parenchyma of this organ after 24 hours' incubation. If the cuboidal epithelium shows activity it is seen in the basal half of the cells, although other lining epithelium of the bronchi and bronchioles appears devoid of enzymatic activity. The basement membrane and the smooth muscle appear strongly active with the muscle nuclei showing the most intense blackening. The smooth muscle of blood vessels is darkened and here again the nuclei more so than the fibers. Blood vessel endothelium, especially the nuclei, show distinct activity. Throughout the entire lung the elastic fibers are stained.

Skin (sole). The enzyme attacking ATP at pH 9.9 shows strong activity after 4 hours' incubation in the stratum Malpighi, especially in the germinativum. Cell nuclei are very strongly active, and in parts the cytoplasm of the cells of the germinativum is so strongly stained that the nuclei can no longer be distinguished. A number of cell nuclei and whole cells, which may be fibroblasts, are blackened in the derma. Blood vessels of all sizes show strong activity with capillaries being stained throughout the skin. The sweat glands are diffusely darkened. Nerves show intense activity in the epineurium, endoneurium and in Schwann's sheath where occasional nuclei can be seen. Cartilage cells show more intense activity in the nucleus.

With ATP as substrate at pH 8.25 after 22 hours' incubation the stratum Malpighi also shows a heavy deposit and cell nuclei in the derma show darkening. The smooth muscle of larger and medium size blood vessels shows activity, especially in the nuclei. The endothelium of larger blood vessels appears darkened, but very little capillary activity can be observed. Cartilage cells show strong blackening of the cell walls and nuclei. Glandular duct cells show intense nuclear activity.

The enzyme attacking A-5-P in 24 hours shows an almost identical distribution with that produced when ATP is used as substrate at pH 9.9 in shorter incubation periods. The only discernible difference in staining is that the blood vessel activity is less pronounced in the deeper layers of the derma.

A-3-P as substrate yields the following picture after 24 hours' incubation: the stratum Malpighi shows slight activity with the germinativum staining the darkest. The cell membranes and nuclei in the germinativum are darker than the cytoplasm. Some small and larger blood vessels as well as capillaries show patchy darkening. Sweat glands show strong activity. After 48 hours' incubation duct cells show blackening, and there is a more pronounced capillary staining which still only appears in parts of a section. Nerves show activity in the endoneurium and epineurium; occasional cell nuclei in the derma show activity also. Here again the cartilage is stained.

With Cr-P at pH 9.9 after two hours' incubation a slight darkening of the sweat glands and the stratum germinativum can be observed. With 4 hours' incubation glandular ducts also show some enzymatic activity. After 48 hours the sweat glands are black with the nuclei and cell membranes darker than the cytoplasm. The stratum Malpighi now shows a slight diffuse darkening. Medium and large blood vessels are slightly darkened, and the nerves show slight and patchy activity in the endoneurium, epineurium and Schwann's sheath.

The enzyme attacking Cr-P in the presence of Mg^{++} at pH 8.9 shows the same distribution as described above at pH 9.9, the only difference being that after 48 hours' incubation the stratum Malpighi shows considerably more activity. Fat cell membranes are darkened, and the endothelium of blood vessels is distinctly active in some parts of a section.

Skin (tail). With ATP as substrate at pH 9.9 after one hour's incubation, a distinct stain of the capillary wall is seen, as well as staining of the endothelial cell membranes. The intima of medium and large blood vessels is very active. The stratum corneum and the cell nuclei of the hair matrix are blackened; the glassy membrane of the hair follicles also shows activity. The nuclei of the epithelium of sebaceous glands and their duct cells are darkened. After an incubation period of three hours the cell nuclei of the stratum Malpighi show an intense activity as well as nuclei of fat cells. In some parts of a section the nuclei of smooth muscle show activity. With 5 hours' incubation the stratum Malpighi appears black, the whole cell in this case being stained darkly. The media of the blood vessels is now darkened together with nuclei of other smooth muscles. The hair follicles are stained darkly, especially the cells of the hair matrix. The basement membrane of sebaceous glands is now very active. Except for the nerve axons, all other parts of the nerves in this location are now seen to be stained. Occasional connective tissue nuclei appear to be blackened.

With ATP as substrate at pH 8.25 very little activity can be observed with less incubation time than 6 hours. After 6 hours the picture is very similar to ATP at pH 9.9. The capillary stain is less pronounced and more irregular. Muscle nuclei are clearly stained wherever smooth muscle can be found in the skin or blood vessels. Endothelial nuclei show strong activity. After 22 hours nerves appear blackened as well as interstitial cell nuclei and fat cell membranes.

With A-5-P as substrate the same enzymatic activity can be observed in 24 hours' incubation as with ATP at pH 9.9 in 5 hours.

With A-3-P as substrate after 24 hours' incubation the nuclei of the sebaceous gland epithelium show strong activity. Capillaries and nerves appear blackened, but only in parts of a section. With 48 hours' incubation the cell walls of the sebaceous gland epithelium now are darkened. Fat cell membranes, hair papillae, and matrix are all active. The stratum Malpighi shows a diffuse blackening and in the germinativum the cell nuclei can be seen.

With Cr-P at pH 9.9 after 48 hours a faint darkening of the sebaceous glands as well as a faint diffuse darkening of the stratum Malpighi can be observed. The stratum corneum and disjunctum are blackened with some activity also seen in the hair matrix.

With Cr-P at pH 8.9 in the presence of Mg^{++} after 48 hours' incubation sebaceous glands show activity, the cell walls and nuclei being blackened. In 72 hours the nuclear membranes of the cells of the stratum Malpighi are darkened.

Heart. With ATP as substrate at pH 9.9 after 4 hours' incubation the following structures show strong activity: central nuclei, endothelium of blood vessels, myofibrils, and the septa between the muscle bundles.

With ATP as substrate at pH 8.25 after 22 hours' incubation the central nuclei are again stained with the nuclear membrane darkened and the chromosomal detritus appearing black with a light zone interposed. The endothelium of large and medium blood vessels, as well as the media are active with muscle nuclei staining in the latter. Myofibrils are blackened and some spiral shaped fibers, as yet unidentified, are strongly active. When glutathione (2×10^{-2} M final concentration) replaces a part of the water of the incubation mixture, the same enzymatic activity can be achieved in three hours' incubation time.

A-5-P as substrate yields in 24 hours' incubation the same picture as ATP at pH 9.9 in 4 hours' incubation time.

With A-3-P as substrate after 24 hours' incubation the central nuclei show activity as well as the endothelium of blood vessels of all sizes. In 30 hours the septa between muscle bundles are blackened, the muscle of larger blood vessels shows activity, and capillaries appear dark. Myofibrils are blackened and cross striation can be seen.

With Cr-P at pH 9.9 after 48 hours' incubation a distinct stain of the myo-septa can be observed. Occasional intercalated disks show darkening. The intima and media of larger vessels are active, capillaries are blackened, and central nuclei show darkening in parts of a section. In other parts of the section, the muscle syncytium is diffusely active.

With Cr-P at pH 8.9 in the presence of Mg^{++} a diffuse muscular stain can be observed in 24 hours with occasional activity seen in the central nuclei. After 48 hours' incubation the central nuclei show distinct activity and the whole muscle appears diffusely blackened.

Digestive system — stomach (pylorus). With ATP as substrate at pH 9.9 after one hour's incubation a strong blood vessel stain can be observed, with capillaries blackened in the lamina propria. The muscularis mucosae appears diffusely darkened. After two hours the lamina propria is considerably darker than the lining epithelium which is separated from the former by a clear unstained zone. After 4.5 hours the nuclei of the smooth muscle are stained darkly; the submucosa appears free of stain except for a few light staining nuclei of interstitial cells. The lining epithelium of the gastric pits shows strong enzyme activity especially at the bottom of the pits. Here cell membranes are distinctly blackened with a fine granular stain appearing in the cytoplasm. A light zone midway in the pits appears to contain cells devoid of cytoplasmic activity. The capillaries at the bottom of the pits are very active.

With ATP as substrate at pH 8.25 after 4 hours' incubation a slight darkening of the muscularis mucosae can be seen. The epithelium lining the inner half toward the bottom of the pits shows considerable activity in the cytoplasm and cell membranes. The basement membrane of the gastric glands is blackened. A patchy blood vessel stain can be observed. Even after 22 hours' incubation the smooth muscle appears only slightly active with the nuclei showing the most activity. The inner two-thirds of the gastric pits show intense enzymatic activity of the whole cells of the lining epithelium.

The enzyme attacking A-5-P in the pylorus shows a similar distribution after 24 hours' incubation as ATP at pH 9.9 in 4.5 hours, except that the muscular and capillary stain is considerably less pronounced.

With A-3-P as substrate after 24 hours' incubation the lining epithelium appears intensely blackened; about midway in the pits a lighter zone can be observed. After 48 hours the muscularis mucosae is stained darkly whereas the muscularis externa is much lighter. With the same period of incubation the muscular media of blood vessels shows intense activity. Capillaries in the interstitial tissue and the endothelium of larger size vessels are also active. Occasional nuclei can be seen in the blackened lining epithelium.

With Cr-P at pH 9.9 after 24 hours' incubation the inner circular and outer longitudinal muscle appear diffusely darkened. The lining epithelium shows activity especially in the inner half toward the bottom of the gastric pits. Here the cell membranes are found blackened. With 48 hours' incubation large cells giving the appearance of parietal cells are stained very darkly, especially the cell membranes. Other lining cells show less activity of the cell membrane. Strong activity is found in the circular muscle layer. The same grading of enzymatic activity from the bottom to the top of the pits is seen as that previously described for ATP.

With Cr-P at pH 8.9 in the presence of Mg^{++} the same pattern of enzyme activity can be observed after 48 hours as with Cr-P at pH 9.9.

Cardia. With ATP as substrate at pH 9.9 after 4 hours the muscular coats appear diffusely darkened with the capillaries blackened in the muscularis mucosae. The muscularis mucosae appears considerably more active than the externa. Blood vessels of all sizes show activity in the endothelium and muscular media. No epitheliial staining can be observed even after 48 hours' incubation.

With ATP as substrate at pH 8.25 only a slight darkening of the muscularis mucosae can be elicited in 4 hours. After 22 hours' incubation, however, this layer appears intensely blackened. Muscle nuclei are darkly stained wherever smooth muscle is found in this organ, and many cell nuclei in the interstitial tissue are stained. The muscular media is active in the large and medium sized vessels.

After 24 hours' incubation with A-5-P as substrate, there is a similar enzyme distribution as with ATP at pH 9.9 in 4 hours. However, occasional fibers show activity in the lamina propria.

The enzyme splitting A-3-P shows activity after 24 hours in the endothelium and muscle of blood vessels of different sizes. All the muscular coats of the cardia are diffusely darkened. After 48 hours the muscularis mucosae and externa appear stained, although this darkening is not as great in the latter layer. The epithelium is diffusely darkened and the capillaries show a heavy deposit.

Cr-P at pH 9.9 after 48 hours' incubation yields enzymatic activity in the muscle fibers and the lining epithelium. The basement membrane fibers are darkened near the cardia-pyloric junction.

With Cr-P at pH 8.9 in the presence of Mg^{++} after 48 hours' incubation all the muscular coats appear strongly active, with the muscularis mucosae being darker than the externa. The blood vessel endothelium shows a distinct stain. The epithelium of the cardia shows only slight activity.

Duodenum. With ATP as substrate at pH 9.9 a very dark stain of the striated border can be seen with two hours' incubation. The nuclei of the lining epithelium of the crypts show activity, as well as the nuclei of smooth muscle and Brunner's glands. Some cell nuclei in the core of the villi are also stained together with the blood vessels located there.

With ATP as substrate at pH 8.25, after two hours' incubation, the same stain that was seen with ATP at pH 9.9 is found. With 4 hours' incubation considerable diffusion into the core of the villi can be observed. The central lacteals are blackened.

With A-5-P as substrate after two hours' incubation a light stain in the nuclei and basal part of the epithelial cell can be observed with the striated border staining intensely. The core of the villi appears light with the central lacteals blackened. Blood vessels of all sizes are distinctly active.

With A-3-P as substrate after one hour's incubation the striated borders of the epithelial cells are blackened while the cytoplasm is only diffusely darkened. A slight diffusion into the core of the villi can be seen, and occasional nuclei of this interstitial tissue show activity. The smooth muscle appears faintly but diffusely darkened, while the capillaries in this region are distinctly active.

With Cr-P at pH 9.9 after one hour's incubation the epithelial basement membrane of the crypts of Lieberkühn is blackened. With two hours' incubation a diffuse darkening of the external muscular coat is seen. With 4 hours' incubation the striated border appears blackened while the rest of the epithelial cell is only diffusely darkened. After 22 hours the epithelial lining shows an intense staining of the apical portion of the cell next to the lumen. There occurs a light zone between this dark apical portion and the inner, less active basal portion of the cell. Occasional light staining cells can be seen in the core of the villi. The muscle is still diffusely darkened and Brunner's glands show intense activity.

With Cr-P at pH 8.9 in the presence of Mg^{++} the striated border of the epithelial cells shows distinct staining after 4 hours' incubation, while the cytoplasm remains only slightly darkened. After 22 hours the muscular coats appear much darker than with Cr-P at higher pH, and the nuclei of this muscle are very active.

Colon. With ATP as substrate at pH 9.9 after 5 hours' incubation the nuclei of the epithelium lining the crypts of Lieberkühn stain intensely, while the striated border appears only slightly darkened. The muscularis mucosae stains strongly and the nuclei of the taeniae coli are very active. Blood vessels of all sizes appear darkened in their endothelium and muscular media. The capillaries located in the muscle as well as in the submucosa are strongly active.

With ATP as substrate at pH 8.25 the epithelial cells are darkened in the apical one-third with the cell membranes of these epithelial cells also showing activity. Staining of the basement membrane of the crypts of Lieberkühn is quite outstanding. The capillary network is active in most parts of the section, including the taeniae coli. With 22 hours' incubation all nuclei of the lining epithelium and smooth muscle appearing in the section show intense staining. The muscularis mucosae shows a diffuse staining of the muscle fibers although the taeniae coli appear darker.

With A-5-P as substrate after 24 hours the same picture of enzymatic activity is achieved as with ATP at pH 9.9 in 5 hours. In addition, the cell membranes of the goblet cells are darkly stained.

With A-3-P as substrate after 24 hours' incubation the basement membrane appears strongly active, as well as the apical one-third of the lining epithelial cell. The striated border is darker than the rest of the cell. Blood vessels of all sizes show activity in the endothelium and muscular coats. With 48 hours' incubation occasional nuclei in the connective tissue appear active. The muscle nuclei show intense staining; the nuclear membrane and chromosomal detritus show blackening with a clear intermediary zone. Capillaries in the muscular coats show a heavy deposit.

With Cr-P at pH 9.9 after 4.5 hours' incubation a faint staining of the striated border can be observed. In 96 hours the lining epithelium is darkened with the apical part of the cell considerably darker than the basal portion. The muscle appears slightly and diffusely darkened. Capillaries show slight activity as well as the endothelium of larger vessels.

With Cr-P at pH 8.9 in the presence of Mg^{++} after 24 hours' incubation the basement membranes as well as the cell walls of the lining epithelium appear blackened. There is a faint darkening of the muscularis mucosae. After 96 hours the nuclei of the muscularis mucosae and the taeniae coli appear blackened, and the whole cell of the lining epithelium is dark.

Rectum. With ATP as substrate at pH 9.9 after one hour's incubation the blood vessels are stained with the endothelium and muscular portion appearing dark. The muscularis mucosae is diffusely darkened with the muscularis externa stained to a lesser extent. The striated border of the epithelium lining the crypts is strongly stained together with the nuclei of these cells toward the bottom of the crypts. With two hours' incubation the nuclei of smooth muscle appear darkened. In 5.5 hours all the nuclei of the epithelium lining the crypts are stained. The submucosa remains unstained, but some nuclei of connective tissue cells show enzyme activity. The muscularis mucosae appears too black to distinguish cell components, but in the longitudinal muscle, nuclei can still be clearly observed.

With ATP as substrate at pH 8.25 after 4 hours' incubation there is a darkening of the basement membrane as well as the epithelial cell membrane. The muscularis mucosae and externa are diffusely blackened. With 22 hours' incubation the following activity is observed: dark staining of muscle nuclei, endothelium and muscular part of the blood vessels. The lining epithelium is strongly active with the cell walls of the goblet cells being clearly outlined and their nuclei blackened toward the bottom of the crypts.

The enzyme attacking A-5-P shows the same distribution after 48 hours' incubation as ATP at pH 9.9 shows in 5.5 hours.

A-3-P as substrate yields the following picture in 24 hours' incubation time: the lining epithelium is distinctly active, especially the striated border and the goblet cell membrane. The basement membrane is blackened and both muscular coats show enzyme activity with the nuclei of the externa more active than those of the muscularis mucosae. The smooth muscle of the blood vessels is also diffusely darkened. After 48 hours the cell membrane and occasional nuclei appear darker than the cytoplasm of the lining epithelium, although they all show activity. Muscle nuclei are active throughout the section and some interstitial tissue cells show darkening in the submucosa. The capillaries show activity in the muscular coats.

After 24 hours' incubation the enzyme attacking Cr-P at pH 9.9 shows activity in the epithelial cell membranes and striated border. The muscular fibers appear diffusely darkened. With 48 hours' incubation the epithelial cells show additional staining toward the lumen and the base, with the cell walls being stained intensely.

With Cr-P at pH 8.9 in the presence of Mg^{++} after 96 hours all smooth muscle and muscle nuclei show activity. The lining epithelium is very dark, and the endothelium and muscular media of the blood vessels are again active.

Liver. With ATP as substrate at pH 9.9 after three hours' incubation the nuclei of the liver cells show activity. The intima of the central vein, the endothelium of the sinusoids, and bile canaliculi are blackened with the interlobular connective tissue showing diffuse activity. After 5 hours' incubation the endothelial nuclei of the central vein are stained along with the cell walls of the bile duct epithelium. With 23 hours' incubation the liver cell nuclei are dark with the nucleoli visible as even darker objects. A fine granular black deposit is seen scattered throughout the cytoplasm. The muscle fibers of large vessels are diffusely blackened with their nuclei showing the greatest activity.

With ATP as substrate at pH 8.25 after 22 hours' incubation the liver cell nuclei, nucleoli, and cell walls all show considerable activity, with the cytoplasm of these cells also being active but to a lesser degree. The lining of the sinusoids is darkened especially near the central vein, the endothelium of which is also darkened. The smooth muscle, especially the nuclei, of the other blood vessels seen in the liver is blackened together with the endothelial nuclei of these vessels. However, this activity is often patchy and not to be found throughout a section.

A-5-P as substrate after 48 hours' incubation yields a stain similar to ATP at pH 9.9 after 5 hours' incubation. There are some differences in that the staining of the liver cell membranes and nuclei appears darkest near the central vein. The cytoplasm of these hepatic cells is filled with small dark granules. The intima, media, and adventitia of larger blood vessels show activity; and the sinusoids appear much darker and more diffuse than with ATP.

The enzyme attacking A-3-P in the liver shows activity only in the sinusoid wall. This occurs even after 106 hours of incubation. The other liver tissue is only slightly and diffusely darkened, very similar to the control slides.

Cr-P at pH 9.9 also yields a nearly negative picture. In 48 hours a faint activity of the central vein endothelium and sinusoidal lining can be observed. A very slight darkening of the liver cell cords, interstitial tissue, intima and media of some vessels is suggested.

With Cr-P at pH 8.9 in the presence of Mg^{++} a distinct cytoplasmic stain of the liver cells and stronger staining of the Kupffer cells can be observed after 48 hours' incubation. In 72 hours smooth muscle and its nuclei are active. With 96 hours' incubation the lining of the central vein shows patchy activity and the liver cell nuclei are darkened in some parts of a section.

Urinary system — kidney. With ATP as substrate at pH 9.9 after one hour's incubation the afferent and efferent vessels show activity, as well as the capillary network of the glomeruli. The capsule of Bowman, glomerular epithelium and some nuclei in the capsular epithelium are blackened. A slight diffuse stain is visible in some tubules of the nephron and tubular nuclei are active. After 4 hours the glomeruli are blackened throughout, with the visceral and parietal epithelium being also stained. The basement membrane of the proximal tubules shows activity with tubular nuclei being darkly stained throughout the cortex, and strongest in the medullary half. The basal part of the tubular cells appears blackened along with the brush border of the proximal tubule. A clear zone lies between these two portions. The collecting tubules of the medulla show very slight activity. Endothelial nuclei and the media of blood vessels are darkened.

With ATP as substrate at pH 8.25 after 6 hours' incubation the brush border of the proximal tubules shows activity in the medullary one-third of the cortex. The capsule of Bowman and the nuclei of the parietal and visceral epithelium appear black with the cytoplasm of these cells stained lightly. The basement membrane of the tubules as well as the nuclei are active. The afferent and efferent vessels show activity in some parts of a section, and the endothelium and media of other vessels are blackened. In 22 hours the picture is very similar to ATP at pH 9.9 in 4 hours, but the stain is patchy and only parts of a section show strong activity. As opposed to heart and voluntary muscle, the addition of glutathione shows no influence on the staining of the kidney.

With A-5-P as substrate after 5 hours' incubation the tubular nuclei near the capsule of the kidney show activity. The adventitia of large and medium size blood vessels is blackened along with the capsule of Bowman. The glomeruli are only slightly and diffusely stained with a few visceral epithelial nuclei being darkened. The brush border of the proximal tubule shows activity as well as the tubular basement membrane. In 24 hours the picture is very similar to ATP at pH 9.9 in 4 hours' incubation, but the glomerular stain is much more patchy and less pronounced, and the tubular stain is mainly present in the nuclei and basement membrane.

With A-3-P as substrate after one hour's incubation the brush border of the proximal tubule shows intense activity throughout the cortex with the tubular nuclei being active in the capsular one-third of the cortex. The adventitia of the blood vessels is seen to be strongly stained. After three hours' incubation the whole tubular cell in the medullary third of the cortex is blackened, but the tubular nuclei are active throughout the cortex. After 22 hours Bowman's capsule, the capillary network of the glomeruli, and the endothelium of blood vessels appear strongly active. The tubular basement membrane in the cortex is stained.

With Cr-P at pH 9.9 after 4 hours' incubation the enzymatic activity is identical with A-3-P after one hour except that the tubular nuclei are more active in the medullary third of the cortex. After 22 hours' incubation the whole tubules stain in the medullary third of the cortex but in the capsular two-thirds, whole tubules appear active only in some parts while in other parts only the nuclei of the tubular cells show staining. Blood vessels show intense activity in the media and adventitia. The visceral nuclei of the glomeruli, the capillary network, and Bowman's capsule all are active. In the medulla a slight diffuse tubular stain of the collecting system can be found.

With Cr-P at pH 8.9 in the presence of Mg^{++} after 5 hours' incubation the tubular nuclei show activity in the medullary half of the cortex. The muscular media of the blood vessels shows diffuse activity. After 22 hours' incubation the following structures show strong enzymatic activity: tubular nuclei throughout the cortex, basement membranes, the glomerular capillary network, Bowman's capsule, visceral and parietal epithelial nuclei, the blood vessel endothelium with its nuclei and smooth muscle nuclei.

Bladder. With ATP as substrate at pH 9.9 after one hour's incubation the muscle is diffusely darkened. Blood vessels of all sizes show strong activity in the endothelium and the capillaries, especially those near the epithelium of the bladder, are strongly stained. After three hours the nuclei of the smooth muscle are blackened and the epithelial nuclei near the lamina propria are darkly stained. After 5 hours' incubation the epithelium stains intensely, still appearing darker toward the lamina propria than the lumen. Muscle shows a diffuse fibrillar and distinct nuclear stain; occasional interstitial cell nuclei show activity in the lamina propria.

With ATP as substrate at pH 8.25 after 4 hours' incubation the transitional epithelium appears diffusely darkened. Smooth muscle fibers are diffusely stained with their nuclei most active. The blood vessel endothelium and some nuclei in the interstitial tissue of the lamina propria are darkened.

With A-5-P as substrate after 24 hours the muscle fibers and nuclei are darkly stained. The blood vessels show intense activity in the adventitia and media, and the transitional epithelium is strongly stained in the cytoplasm as well as in the nuclei.

With A-3-P as substrate after 48 hours' incubation capillaries and endothelium of other vessels appear blackened together with some darkening of muscle fibers and nuclei in the media of these vessels. Some interstitial cell nuclei found in the lamina propria also show activity, and the transitional epithelium is diffusely darkened.

Cr-P at pH 9.9 in 24 hours shows a diffuse darkening of the muscle. The transitional epithelium of the bladder is diffusely active with very dark nuclei. With 48 hours' incubation the cytoplasm of the lining epithelium toward the lamina propria is black and occasional cells in the subepithelial layer are darkened.

With Cr-P at pH 8.9 in the presence of Mg^{++} after 48 hours the smooth muscle of the bladder and blood vessels is diffusely blackened. The transitional epithelium also shows this activity. No change in this picture can be observed even after 120 hours of incubation.

Striated muscle (sectioned in the abdominal wall). The enzyme attacking ATP at pH 9.9 shows a slight diffuse activity in the muscle fibers after one hour's incubation. In 24 hours the muscle fibers are blackened with their eccentric nuclei appearing blacker than the other tissue components. The nuclear membrane is also darkened and an unstained zone is interposed between it and the chromosomal detritus. The sarcolemma, capillaries and blood vessel endothelium all appear distinctly active.

With ATP as substrate at pH 8.25 after 22 hours' incubation the muscle fibers, sarcolemma, and chromosomal detritus of the muscle nuclei are stained intensely. Capillaries and the endothelium of blood vessels of all sizes together with the smooth muscle of the media are diffusely darkened. The nuclei of these structures show considerable activity. Nerves show intense staining of the epineurium, endoneurium, and perineurium with the elastic fibers of the latter and the nuclei of the neurolemma outstandingly active. The activity in striated muscle is very markedly enhanced in the presence of glutathione (2×10^{-2} M). With this activator the same intensity of staining is achieved in three hours' incubation as is found in 22 hours' incubation without glutathione.

With A-5-P as substrate, even after 72 hours only a diffuse stain of the muscle fibers can be obtained. In some parts of a section striated muscle nuclei show slight activity, and the adventitia and media of blood vessels are seen to be slightly stained.

With A-3-P as substrate after 96 hours' incubation only a diffuse muscle fiber stain can be observed, but in some parts cross striation can be seen clearly.

With Cr-P at pH 9.9 after 48 hours only a diffuse patchy stain of the striated muscle is seen but the sarcolemma is distinctly active.

With Cr-P at pH 8.9 in the presence of Mg^{++} after 72 hours' incubation a diffuse staining of the muscle is observed, and in parts, a clear nuclear darkening is present. The sarcolemma, however, is not active.

Male genital system — prostate. In three hours' incubation with ATP as substrate at pH 9.9 the acinar epithelium shows darkening, especially toward the connective tissue. The epithelial cell nuclei are stained darkly and some cell nuclei of the interstitial tissue show activity. The smooth muscle of the septa around the acini shows a diffuse cytoplasmic staining with its nuclei appearing still darker. Blood vessels of all sizes show activity in the endothelium, and the larger vessels are also stained in the muscular media.

With ATP as substrate at pH 8.25 after 22 hours' incubation the pseudostratified epithelium shows strong activity in the nuclei with a diffuse slight activity in the cytoplasm. Elastic fibers and smooth muscle nuclei in the interstitial tissue are blackened. The nuclei and cytoplasm of the endothelium in all blood vessels are strongly active.

With A-5-P as substrate after 24 hours' incubation the picture is similar to ATP at pH 9.9 after three hours' incubation except that the acinar epithelium shows no cytoplasmic darkening.

With A-3-P as substrate after 24 hours of incubation the epithelial cell membranes show slight, while the nuclei show strong, enzymatic activity. Some elastic fibers in the cells show slight activity in the cytoplasm combined with a strong nuclear stain. The adventitia of blood vessels shows darkening.

Cr-P at pH 9.9 in 24 hours shows a diffuse stain of the glandular epithelium. Subepithelial fibers are intensely blackened and occasional interstitial cells have activity in their nuclei. The endothelium of blood vessels is stained strongly.

With Cr-P at pH 8.9 in the presence of Mg^{++} after 48 hours' incubation the pseudostratified epithelium shows darkening of the cell membranes, and in some parts of a section the basal portion of the epithelium appears very active.

Coagulating gland. The enzyme present in this gland shows the same distribution for all substrates as in the prostate.

Seminal vesicle. With ATP as substrate at pH 9.9 after two hours' incubation the elastic fibers of the external connective tissue sheet are strongly active. With 5 hours' incubation some interstitial cell nuclei stain, as well as the nuclei of smooth muscle. The endothelium of all sizes of blood vessels is darkened with the smooth muscle of the media of larger vessels showing activity. The nuclei of the glandular epithelium of the basal and cuboidal cells are blackened.

With ATP as substrate at pH 8.25 after 22 hours' incubation the nuclei of the glandular epithelium show activity. The smooth muscle nuclei in the interstitial tissue and the media of the blood vessels are blackened. Elastic fibers in the interstitial tissue together with many interstitial cells are darkened. In some parts of a section the endothelium of large and medium size blood vessels is active.

With A-5-P as substrate a similar picture is achieved in 24 hours as with ATP at pH 9.9 in 5 hours.

With A-3-P as substrate after 20 hours' incubation the nuclei of the glandular epithelium are strongly active as well as elastic fibers in the connective tissue. The intima and media of blood vessels show diffuse activity. With 30 hours' incubation smooth muscle nuclei, capillaries, and interstitial cells are blackened.

With Cr-P at pH 9.9 as substrate after 5 hours' incubation a distinct darkening of elastic fibers in the connective tissue can be observed. In 24 hours the basal layer of the glandular epithelium as well as the fibers in the submucous layer show strong activity. The cytoplasm of the smooth muscle fibers is slightly and diffusely stained, and in some interstitial cells near the epithelium the nuclei show strong activity.

With Cr-P at pH 8.9 in the presence of Mg^{++} after 24 hours' incubation a slight diffuse darkening of the glandular epithelium and smooth muscle fibers can be seen. In 48 hours, epithelial and smooth muscle nuclei are darkly stained. The intima and media of the blood vessels are active, and the capillaries are darkened.

Female genital system — ovary. ATP as substrate at pH 9.9 yields the following picture: after two hours' incubation the nuclei and cell membranes of corpora lutea cells as well as the nuclei of both young and atretic follicle cells are active. The endothelium of blood vessels is stained. After three hours' incubation the nuclei of the follicular cells of growing follicles stain with the cytoplasm slightly darkened. Capillaries and the endothelium and media of larger vessels show strong activity. Some cells in the interstitial tissue are darkened. With 4.5 hours' incubation all follicular and theca cells appear active.

With ATP as substrate at pH 8.25 after two hours' incubation a faint darkening of the theca cells is observed. After 22 hours the follicular as well as theca cells of the Graafian follicle show intense activity and the egg cell is blackened. The basement membrane of follicles as well as the glassy membrane of corpora lutea are darkened. Blood vessels of all sizes show endothelial activity with the smooth muscle nuclei of the media appearing black in comparison to the light staining fibers. Medium and larger vessels show a darkening of the adventitia as well. Numerous cell nuclei in the interstitial tissue show activity. The corpora lutea cells are darkened in the cytoplasm, cell wall and nucleus.

With A-5-P as substrate the same picture is observed after 24 hours' incubation as is seen when ATP at pH 9.9 is used for 4.5 hours.

With A-3-P as substrate after 24 hours' incubation the nuclei, cytoplasm, and cell membranes of follicular cells show activity, although the theca cells are still more active. Corpora lutea cell nuclei are dark with the follicular basement membrane and glassy membrane of the lutea showing intense activity. The egg cell nucleus is intensely blackened. The muscle found in blood vessels shows intense nuclear stain with a slight staining of the muscle fiber. The endothelium of blood vessels is also darkened.

With Cr-P at pH 9.9 after 24 hours' incubation the theca cells show diffuse darkening with the basement membrane of the follicle intensely stained. The corpora lutea cell is also diffusely darkened. The media of blood vessels is active; fibers, as well as nuclei of some cells in the interstitial tissue show activity. With 48 hours' incubation the intima and adventitia of larger vessels have become stained with the nuclei in the media intensely darkened. All theca cells are very black with the follicular cells being much lighter.

Cr-P at pH 8.9 in the presence of Mg^{++} in 48 hours shows activity in the corpora lutea cells, the cell wall and nucleus being darkened. In primary follicles, the egg cell nucleus is very black. The theca and follicular cells in addition to the basement membrane show intense activity, and blood vessels show staining in the intima and media.

Fallopian tube. With ATP as substrate at pH 9.9 after two hours' incubation the epithelium lining the tube shows an intense nuclear stain. The fibers of the lamina propria are blackened and both muscle layers show diffuse activity. Blood vessels and capillaries show intense endothelial activity with the endothelial nuclei distinctly darker. With further incubation the muscle nuclei become darkened and occasional cell nuclei are seen to be active in the lamina propria.

With ATP as substrate at pH 8.25 after 22 hours' incubation the nuclei of the epithelium are intensely active. Blood vessels of all sizes show activity in the endothelium and media, and in the latter, muscle nuclei are particularly black. Occasional cells and cell nuclei in the interstitial tissue appear darkened.

With A-5-P as substrate after 24 hours' incubation blood vessels and capillaries show strong activity of the endothelium. The fibrous tissue of the lamina propria shows activity, but the epithelium lining the tube is completely devoid of any stain.

With A-3-P as substrate with 30 hours' incubation all blood vessels show activity in the endothelium as well as in the medial muscular coat, where the nuclei appear most active. The epithelium shows darkening of the cytoplasm with the nuclei entirely blackened. Occasional interstitial cells appear darkened.

With Cr-P at pH 9.9 after 22 hours of incubation the nuclei of the lining epithelium are strongly active. Blood vessel endothelium, the media of larger vessels, and cell nuclei in the interstitial tissue are darkened.

Essentially the same stain can be elicited with Cr-P at pH 8.9 in the presence of Mg^{++}, although the nuclei of the muscular media seem to be more strongly stained than with Cr-P at pH 9.9.

Uterus. With ATP as substrate at pH 9.9 after 4.5 hours' incubation, the mucous epithelium lining the uterine cavity shows a slight staining of the cytoplasm combined with a strong nuclear and membrane activity. The epithelium and especially the nuclei of the uterine glands, together with the basal part of these cells, are intensely stained. Blood vessels are strongly active in the endothelium and the muscular coat. The luminal membrane of the lining epithelium and many nuclei and whole cells of the interstitial tissue are darkened.

With ATP as substrate at pH 8.25, with 4 hours' incubation a slight darkening of the uterine glands, myometrium, and mucous epithelium can be observed. After 22 hours the nuclei of the mucous epithelium and many interstitial cell nuclei of the endometrium are active. The stellate cells show activity in the cytoplasm and cell membrane. The endothelium of blood vessels, especially the nuclei, are strongly stained. Smooth muscle fibers are darkened with the greatest activity seen in the nuclei of the muscle. The uterine glands are very dark.

A-5-P yields a similar picture after 24 hours' incubation as ATP at pH 9.9 after 4.5 hours. Stellate cells show intense staining, but instead of the luminal membrane of the lining epithelium, the basement membrane shows the activity. Nuclei of the blood vessel endothelium are very active with the cytoplasm being less dark.

With A-3-P as substrate in 22 hours' incubation the enzymatic activity is observed in the following locations: nuclei and cytoplasm of the lining mucosa, nuclei of the uterine gland epithelium, and endothelium of all sizes of blood vessels, especially the nuclei of the latter. Smooth muscle is diffusely but slightly darkened with the greatest concentration of activity in the nuclei. Numerous cells and cell nuclei in the interstitial tissue, as well as the stellate cells, are active.

With Cr-P at pH 9.9 after 4.5 hours a faint darkening of the uterine glands and mucosa can be seen. In 24 hours the blood vessels show staining of the endothelium; the epithelium of the uterine mucosa and uterine glands is active. After 48 hours' incubation the uterine mucosa shows better localized activity, with the epithelial nuclei stained darkly. Glandular duct cells, many interstitial cells and their nuclei, as well as the smooth muscle and its nuclei are very dark.

With Cr-P at pH 8.9 in the presence of Mg^{++} after 48 hours of incubation, epithelial, endothelial, and smooth muscle nuclei are distinctly active. In the uterine glands, activity can be found in the nuclei and cell walls, whereas the mucosa shows activity in the nuclei and cytoplasm. Capillaries and larger vessels are stained throughout the section.

SUMMARY OF HISTOLOGIC FINDINGS

In table 4 the differences in activity and in organ distribution are listed for the substrates studied within the scope of this work. The different substrates showed regularly reproducible differences in the histological activity patterns elecited. The enzyme system attacking ATP at pH 9.9 invariably shows activity in blood vessels of all sizes after short incubation periods. There are many differences between the histological activity pattern for ATP and that for muscle-adenylic acid (striated muscle, etc.), and the latter substrate regularly required much longer incubation than the former. Activity for A-5-P was regularly found in the adventitia of blood vessels. On very prolonged incubation, however, the histological distribution for both these substrates becomes very similar in most of the organs studied. The activity pattern for yeast-adenylic acid, on the other hand, differs grossly from that found with ATP or with A-5-P.

The validity of attributing the splitting of creatine-phosphate to two different enzymes or enzyme systems, one active in the presence of magnesium ion and one in its absence, at a more alkaline pH, is clearly indicated by the results summarized in table 4.

Differentiation of substrate specific enzymes by inhibition and activation. In studying the influences of activators and inhibitors, we have found striking differences in their effects with respect to the different substrates employed. These differences are shown in tables 5 and 6 which summarize the results of numerous experiments.

The inhibitors and activators were regularly tested on kidney sections, but we have also tried their effects on several other tissues, though not on all organs and tissues listed in table 1. If there are organ specific variations among the substrate specific enzymes, this would have escaped our observation. However, no differences in activation or inhibi-

TABLE 4

Summary of distribution of enzyme activity in rat tissues with ATP, A-5-P, A-3-P, and Cr-P ± Mg as substrates

ORGANS AND TISSUES	ATP pH 9.9	ATP pH 8.25	A-5-P	A-3-P	Cr-P pH 9.9	Cr-P pH 8.9 + Mg^{++}
Brain	+	+	+	+	+−	+
Lung	+	+	+	+	+	+
Skin (tail)	+	+	+	+	+−	+
Skin (sole)	+	+	+	+−	+	+
Heart	+	+	+	+	+−	+
Pylorus	+	+	+	+	+	+
Cardia	+	+	+	+	+	+
Duodenum	+	+	+	+	+	+
Colon	+	+	+	+	+	+
Rectum	+	+	+	+	+	+
Liver	+	+	+	+−	+−−	+
Kidney	+	+	+	+	+	+
Urinary bladder	+	+	+	+	+	+−−
Striated muscle	+	+	+−	+−	+−	+−
Prostate	+	+	+	+	+	+
Seminal vesicle	+	+	+	+	+	+
Coagulating gland	+	not checked	+	+	+	+
Ovary	+	+	+	+	+	+
Fallopian tube	+	+	+	+	+	+
Uterus	+	+	+	+	+	+

+ = strong activity, well localized.

+− = slight activity, or patchy and diffuse activity.

+−− = hardly any activity, slightly darker than controls.

CONTROL [1]	INHIBITOR OR ACTIVATOR [2]	SUBSTRATES											
		ATP pH 9.9		ATP pH 8.25		A-5-P pH 9.9		A-3-P pH 9.9		Cr-P pH 9.9		Cr-P pH 8.9 $MgCl_2$	
		Hrs. incubated	Staining intensity	Hrs. incubated	Staining intensity	Hrs. incubated	Staining intensity	Hrs. incubated	Staining intensity	Hrs. incubated	Staining intensity	Hrs. incubated	Staining intensity
—	—	1 4	+ ++	6 22	+ ++	5 24	+ ++	1 22	+ ++	4 22	+ ++	5 22	+ ++
—	Na_2HAsO_4 10^{-2} M	1 4	+ ++			5 24	+— ++	3 22	+— ++	4 22	+ ++	5 24	+— +—
—	$NaAsO_2$ 10^{-2} M	1 4	+ ++			5	++	1	++	4	++	5 24	+ ++
—	Iodoacetate 10^{-2} M	4 20	+— ++	6 24	+— +	5 24	+ ++	1 22	+ ++	4 24	+— +	5 24	+— ++
—	Semicarbazide 1.6×10^{-2} M	4 20	+— ++			5 24	+ ++	1 22	+ ++	4 24	— +—	5 24	— +
Slightly darkened	$CuCl_2$ 10^{-3} M	1 4	+ ++D	6 22	+ ++D	5	++	3 22	+ ++	4 24	+— +	5 24	+ ++
—	H_2O_2 10^{-3} M	2 4	+— +			5	++						
—	Glutathione 2.5×10^{-2} M	2	++	6 22	+ ++	5 24	+— +			4 22	+— ++	24 48	— —
—	p-chloromercuri benzoate 2.5×10^{-4} M	4 22	+— +—	6 24	+— +—	5 24	+— +—	1 22	+ ++	4 24	+— +	5 24	+— +
—	$MgCl_2$ 1.35×10^{-2} M	4	++	6 22	+ ++			1	++			5	++
—	Iodoacetamide 2×10^{-2} M	4 22	— +—			5 24	+— +	1	++	4 24	— —	5 24	— +

[1] No substrate, or boiled organ and substrate.

[2] Molarity given as final concentration in incubation mixture.

—	no activity.
+—	slight activity.
+	activity.
++	intense activity.
D	diffuse activity.

tion have been found among the organs compared except for ATP at pH 8.25. In heart muscle and striated muscle activity for ATP at pH 8.25 was markedly inhibited by sulfhydryl binders and markedly activated by glutathione. Cu^{++} shows only a slight inhibitory effect in our experiments due to complex formation with the glycine present in the buffer (Bailey and Perry, '47). The ATP-ase inhibitors described in the literature, i.e., iodacetate and iodoacetamide,[6] the specific sulfhydro inhibitor, p-chloromercuri benzoate,[7] show the expected reactivity in heart and striated muscle (Laki, '43; Barron and Singer, '45). Magnesium ion shows only slight inhibitory action, even at a concentration of 1.35×10^{-2} M. This may be attributed to the overwhelming

TABLE 6

Activation of myosin ATP-ase by glutathione

ORGAN	CONTROL[1]	INHIBITOR OR ACTIVATOR[2]	SUBSTRATES			
			ATP pH 9.9		ATP pH 8.25	
			Hrs. incubated	Staining intensity	Hrs. incubated	Staining intensity
Striated muscle	3 hrs.: — 24 hrs.: diffuse darkening	—	24	++	22	++
Striated muscle	—	Glutathione 2.5×10^{-2} M	24	++	1 3	+ ++
Heart	24 hrs.: slight diffuse darkening	—	4	++	22	++
Heart	—	Glutathione 2.5×10^{-2} M	4	++	1 3	+— ++

[1] No substrate, or boiled organ and substrate.

[2] Molarity given as final concentration in incubation mixture.

+— slight activity.

+ activity.

++ intense activity.

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[6] See footnote 4, p. 400.

[7] See footnote 4, p. 400.

concentration of calcium ion present in our incubation mixture. We believe, therefore, that ATP at pH 8.25 is split by the classical sulfhydro-sensitive enzyme (Szent-Gyorgyi's myosin ATP-ase) in heart and striated muscle (Banga and Szent-Gyorgyi, '43; Potter, '44; Barron and Singer, '45; Zeller, '48). In kidney, brain and bladder the sulfhydryl inhibitors tested had some but glutathione had no influence on the activity for ATP at pH 8.25. In these and also in other organs activity for ATP was often found to be spotty and irregular. It is clear that activity for ATP at pH 8.25 in tissues other than cardiac and voluntary muscle is due to an enzyme system which is not the same as myosin ATP-ase. The histological activity pattern for ATP at pH 8.25 in these organs resembles, though it does not exactly duplicate, that found for this same substrate at pH 9.9. The response to activators and inhibitors at these two different pH levels is also similar. Consequently, the activity in these organs at these two different pH levels may, perhaps, be due to the same enzyme. It should be pointed out, however, that in all organs activity for ATP at pH 9 to 9.4 is extremely feeble, much more feeble than either at pH 9.9 or at pH 8.25.

The experimental results with inhibitors and activators do not yield an identical picture for the enzymes or enzyme systems which hydrolyze ATP and muscle-adenylic acid at pH 9.9. Arsenate, which slightly inactivates the A-5-P splitting catalyst, and arsenite which slightly activates it, show no influence on the cleavage of ATP at this pH. On the other hand, iodoacetate and semicarbazide slightly inhibit action on ATP but do not interfere with the splitting of A-5-P. Iodoacetamide and p-chloromercuri benzoate inhibit the reaction with both of these substrates, whereas glutathione slightly activates ATP in the kidney at pH 9.9 and inhibits hydrolysis of A-5-P. In constrast to ATP at pH 8.25, this substrate at pH 9.9 is not activated by glutathione either in striated muscle or in the heart.

The only compound found to inhibit partially the enzyme splitting yeast-adenylic acid was arsenate, while arsenite, iodoacetamide and magnesium ion activated it. It is possible that the activation with iodoacetamide may be connected with the destruction of an endogenous inhibitor.

The two enzymes or enzyme systems dephosphorylating creatine-phosphate, one active at pH 8.9 and requiring magnesium ion for its activity, the other active at pH 9.9 in the absence of magnesium, can also be clearly differentiated by the results of the inhibition and activation experiments. Glutathione fully inhibited the Mg^{++} activated enzyme system, whereas it displays a fleeting influence on the other. The failure of glutathione to affect the system acting at high pH might be attributed to the rapid auto-oxidation of glutathione at this pH. However, glutathione was found to be an effective inhibitor for the enzyme system which hydrolyzes A-5-P at the same high pH.

Cupric chloride shows no influence on the Mg^{++}-activated moiety, which is slightly inhibited by arsenate, whereas the other enzyme system is slightly inhibited by Cu^{++} and not affected by arsenate. Iodoacetate, semicarbazide, p-chloromercuri benzoate and iodoacetamide are inhibitors common to both enzymes.

DISCUSSION

1. *Myosin ATP-ase*

The biochemical literature in the field of adenosine-triphosphate dephosphorylation is vast, and a number of ATP-splitting enzymes have been found in different tissues (Potter, '44; Libet, '47; Zeller, '48). Variations in enzymatic activity in the organs have been attributed to differences in calcium and myokinase concentrations present in those organs (DuBois and Potter, '43). Some organs have also been classified according to a gradient of endogenous enzymatic activity for ATP (Engelhardt, '46). Much work on the relation of myosin and acto-myosin to ATP-ase has been done by Szent-Gyorgyi and his school (Banga and Szent-Gyorgyi, '43; Laki, '43; Banga, '47; Szent-Gyorgyi, '47, etc.). Bailey's studies ('42, '44) substantiate the identity of myosin with ATP-ase.

This identity was checked by comparing enzyme activity and birefringence of flow. Studies were made to determine if oxidative reagents, alkylating reagents, and mercaptide forming reagents showed parallel influence on these two qualities Barron and Singer, '43, '45; Singer and Barron, '44; Binkley, Ward and Hoagland, '44; Ziff, '44; Mehl, '44, Bailey and Perry, '47; Roche, Thoai and Bernard, '49). Most authors found that the specific mercaptide former, p-chloromercuri benzoate, was the most pronounced inhibitor for both myosin properties and concluded that ATP-ase activity is an intrinsic property of the myosin molecule and is intimately linked with the free sulfhydryl groups of that molecule. It is of interest that all of these studies on myosin ATP-ase have been concerned with striated muscle and that no similarly detailed studies have been made on smooth muscle. Our histochemical findings strongly indicate that the ATP-ase of smooth muscle is different from that in voluntary and cardiac muscle.

The threshold of inactivation of myosin ATP-ase with magnesium ion and of activation with calcium ion varies with different authors (Gordon, '49). This variance is attributed to differences in protein concentration employed in the experiments and to differences in endogenous salt concentrations and myokinase activity in the tissues.

When using the inhibitors and activators tested by others in *in vitro* studies on purified myosin, we find that these agents have to be used in higher concentrations for histochemical work. Since we have performed our experiments with a limited volume of fluid, it may be that competition amongst the many sulfhydryl groups in the tissue diminishes the effective concentration of the sulfhydryl binders that we have used. It is possible, however, that in the relatively undisturbed state of the fresh frozen tissue sections the sulfhydryl groups of myosin ATP-ase are less accessible to attack than they are in purified myosin solutions. Since our experiments were performed aerobically, oxidation of glutathione undoubtedly occurs — both auto-oxidation (especially at higher pH) and enzymatic oxidation by the respiratory enzymes of the tissue sections. The effective concentration of residual glutathione in our substrate solutions is, therefore, no doubt much less than that at which we have set up these experiments.

The inhibition of activity hydrolyzing ATP at pH 8.25 in heart and striated muscle by p-chloromercuri benzoate at a concentration of 2.5×10^{-3} M is complete, and after exposure to this inhibitor for half an hour activity cannot be restored by treatment with 2×10^{-2} M glutathione. Shorter exposures to the inhibitor were not studied.

In view of all the preceding discussion we conclude that the enzyme which we find active against ATP in voluntary and cardiac muscle at pH 8.25 is myosin ATP-ase. It is of interest that within striated muscle no distinctive localization of this enzymatic activity could be demonstrated in our preparations in relation to the cross striations of the muscle fibers. It should be noted also that the ATP-ase which we find in smooth muscle differs from the myosin ATP-ase both in its pH optimum and in its response to activators and inhibitors.

2. Other enzymes which hydrolyze ATP and muscle-adenylic acid (A-5-P)

The activity for ATP at pH 8.25 in the kidney is not increased by the presence of glutathione and is only partially inhibited by p-chloromercuri benzoate. We conclude that this activity is caused by an enzyme different from that found in heart and voluntary muscle. The question arises as to whether this activity is to be attributed to the same enzyme that attacks ATP at pH 9.9. The anatomical distribution outside of heart and voluntary muscle is similar, though not identical, at the two pH levels studied. In the kidney the reaction to inhibitors is similar at both pH levels. On the other hand, activity in the kidney is greater at pH 8.25 and 9.9 than it is at pH 9.0, and the assumption that the same enzyme is active at pH 8.25 and 9.9 involves the assumption that this system has two separate pH optima.

A puzzling feature is the fact than under some conditions the staining of the sections was very patchy and uneven. This was particularly true in regard to ATP at pH 8.25 in organs other than voluntary muscle and heart. In part such patchy staining may be due to the accidental folding of sections lying on the bottom of the dish or to the accidental covering of one section by another so that the components of the substrate solution do not reach all areas of all sections equally. Alternatively, the tissue sections may contain a necessary cofactor or endogenous inhibitor which is unequally lost from different portions of the sections. Finally, it is possible that these local variations in enzymatic activity may represent real physiological variations within the tissue. Holmgren and Svanborg ('50) showed variations in the enzymatic activity of the liver related to the time of day at which the liver was excised. It must not be understood that patchy distribution of activity has been a frequent or regular finding in our studies. The occasions when we have seen this have been noted above in the section dealing with histological findings.

When using ATP as substrate at pH 9.9 we find that this compound is much more rapidly dephosphorylated than A-5-P, which substantiates Liebknecht's findings ('39, '40) that the phosphate group of adenylic acid is most difficultly hydrolyzable. On prolonged incubation with ATP at pH 9.9 the final pattern of distribution of the reaction product in the sections resembles closely that found on prolonged incubation with A-5-P as substrate, although the deposit from ATP is often so dense after long incubation that histological details are obscured. It is probable that at this pH we are encountering a two stage dephosphorylation of ATP, namely, one which leads to adenylic acid, and a second one which splits the last phosphate from adenylic acid. The latter enzyme might be a specific 5-nucleotidase system (Reis, '34, '37; Gulland and Jackson, '38; Newman et al., '50), while the enzyme responsible for stage one might be a less specific pyrophosphatase (Folley and Kay, '36) or a specific ATP-ase. Also some of the inhibitors (Na_2HAsO_4, iodoacetate, semicarbazide) tested with the two substrates showed slight differences, but these differences disappeared on prolonged incubation. Hydrogen peroxide (10^{-2} M) and $NaAsO_2$ (10^{-2} M) activate the 5-nucleotidase, although the former slightly inactivates and the latter shows no influence on the first stage of the dephosphorylation of ATP. When the first stage is inhibited, the 5-nucleotidase does not find a substrate to act upon. Glutathione (2.5×10^{-2} M), on the other hand, activates the first stage in the splitting of ATP in the kidney while it slightly inhibits the 5-nucleotidase. The dephosphorylation of ATP by one enzyme to adenylic acid in liver extracts was demonstrated by Kalckar ('44) in *in vitro* work. He also showed that in striated muscle, both the action of ATP-ase and myokinase were responsible for the breakdown of ATP (Kalckar, '43, '44). We find that glutathione shows no influence in striated muscle (which is devoid of 5-nucleotidase) when ATP is used as a substrate at pH 9.9. Therefore, it is most unlikely that the activity in muscle at high pH is attributable to the Szent-Gyorgyi enzyme. In embryonic tissue, Steinbach and Moog ('45) succeeded in separating ATP-ase from an apyrase by differential centrifugation. Iodoacetamide (2×10^{-2} M) and p-chloromercuri benzoate (2.5×10^{-4} M) are inhibitors for both stages of ATP dephosphorylation at pH 9.9.

ATP-ase activity at both pH 9.9 and 8.25 was found in all the organs studied and the activity was sharply localized in relation to the tissue structures. The localization patterns were similar at the two pH levels tested. In general the activity was much more intense at the higher pH. While cells and tissues of all types exhibiting activity, the most intense activities, i.e., the earliest appearing and heaviest deposits, were found in smooth muscle and vascular endothelium.

3. *Yeast-adenylic acid (A-3-P)*

Levine and Dillon ('30, '32) in *in vitro* studies tried to separate nucleotidase from nucleosidase activity in the rat's intestine using A-3-P and A-5-P as substrates and found a non-specific phosphatase which they described as a nucleotidase. This enzyme was slightly inhibited by arsenate (10^{-2} M) and dephosphorylated A-3-P preferentially. Possibly this is the enzyme that attacks A-3-P in our histochemical preparations. Iodoacetate (10^{-2} M) (Bradfield, '50), p-chloromercuri benzoate (2.5×10^{-4} M), or iodoacetamide (2×10^{-2} M) do not show any inhibitory capacities in our histochemical work on this substrate. The latter agent even activates the enzyme or enzyme system hydrolyzing A-3-P, possibly by the destruction of an inhibitory substance present in frozen sections. This enzyme is strongly activated by magnesium ion which is in accordance with most *in vitro* studies on "non-specific" alkaline phosphatase. The literature on this subject has been recently reviewed by Roche and Thoai ('50). On grounds of organ and tissue distribution, as well as inhibition and activation experiments, we believe that the enzyme or enzyme system hydrolyzing A-3-P is distinct from that attacking A-5-P.

4. *Creatine-phosphate (Cr-P)*

We have been able to differentiate two enzymes in the rat's body hydrolyzing creatine-phosphate, one active in the presence, and the other at a slightly more alkaline pH in the absence of magnesium ion. Ichihara ('33) synthesized several organic compounds with an N-P linkage which were split in the kidney at an optimal pH of 9. He succeeded in separating alkaline phosphatase from an enzyme hydrolyzing specifically N-P linkage which he called phosphoamidase. Whether this phosphoamidase is identical with the enzyme which we find active in the absence of magnesium can only be decided by further study. The magnesium activated enzyme might be identical with a "non-specific" alkaline phosphatase studied by Winnick ('47) *in vitro* with creatine-phosphatase as substrate. A kinetic gradient of dephosphorylation by alkaline phosphatase using different substrates was established by Waldschmidt-Leitz and Koehler ('33). This gradient also holds for our histochemical work. We find that arsenate (10^{-2} M) partially inhibits the magnesium activated enzyme but does not affect the enzyme active at pH 9.9 in the absence of magnesium ion. On the other hand, $CuCl_2$ (10^{-3} M) partially inactivates the latter but not the former. Glutathione (2.5×10^{-2} M), as noted previously, completely destroys activity of the Mg-activated system, suggesting that a heavy metal may be necessary for this enzyme. Iodoacetate (10^{-2} M) is more inhibitory for the phosphoamidase active at pH 9.9; semicarbazide (1.6×10^{-2} M), p-chloromercuri benzoate (2.5×10^{-4} M) and iodoacetamide (2×10^{-2} M) are inhibitors common to both enzymes. The previously discussed differences in organ distribution, and in susceptibility to inhibition and activation, make it obvious that two enzymes or enzyme systems are active in the dephosphorylation of creatine-phosphate. Fell and Danielli ('43) and Danielli, Fell and Kodicek ('45) in histochemical studies on alkaline phosphatase using pathological materials, alcohol-fixed and paraffin-embedded, found changes in a non-specific alkaline phosphatase associated with fibrillar proteins. The enzyme active on Cr-P at pH 9.9 in our study often shows staining of the fibrous material in interstitial tissue and basement membranes. Perhaps the most noteworthy anatomical finding with creatine-phosphate as substrate is the almost complete absence of activity for this substrate in striated muscle under the conditions of our experiments. In view of the long established evidence that striated muscle can utilize creatine-phosphate as a source of high energy phosphate, we are forced to conclude that the enzyme responsible for the hydrolysis of this substrate in muscle must be a phosphotranspherase incapable of attacking creatine-phosphate in the absence of the appropriate phosphate acceptor.

SUMMARY

1. It is possible to demonstrate a number of enzymes or enzyme systems active in the alkaline range on adenosine-triphosphate, muscle- and yeast-adenylic acids, and creatine-phosphate as substrates, using fresh frozen rat tissue sections with a modification of the histochemical alkaline phosphatase technique of Gomori ('39)-Takamatsu ('39). These enzyme systems can be differentiated from one another by their

different localization in the tissues and by their different responses to activators and inhibitors.

2. The activity of myosin ATP-ase was demonstrated histochemically in striated muscle and heart using adenosine-triphosphate as substrate at pH 8.25.

3. The dephosphorylation of adenosine-triphosphate at pH 9.9 in all tissues of the rat except striated muscle probably involves a two stage enzymatic process. The first stage is attributed to a pyrophosphatase, or ATP-ase, which splits this substrate to muscle-adenylic acid.

4. A specific 5-nucleotidase was demonstrated histochemically in the rat tissues when muscle-adenylic acid was used as substrate. This enzyme presumably catalyzes the second stage of ATP dephosphorylation.

5. Yeast-adenylic acid is hydrolyzed in fresh frozen tissue sections by an enzyme which differs in its anatomical distribution and in its response to activators and inhibitors from the enzyme which attacks muscle-adenylic acid.

6. It was shown that creatine-phosphate is hydrolyzed by two enzymes or enzyme systems, one active in the presence of magnesium ion and one in its absence at a more alkaline pH.

We are indebted to Mr. Delbert Parker for the photomicrographs.

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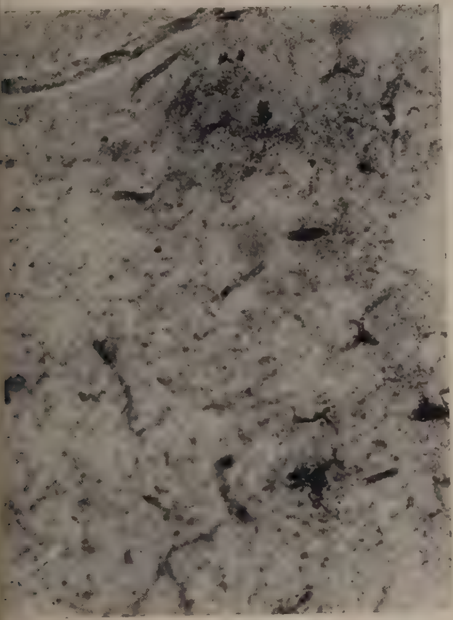
PLATES

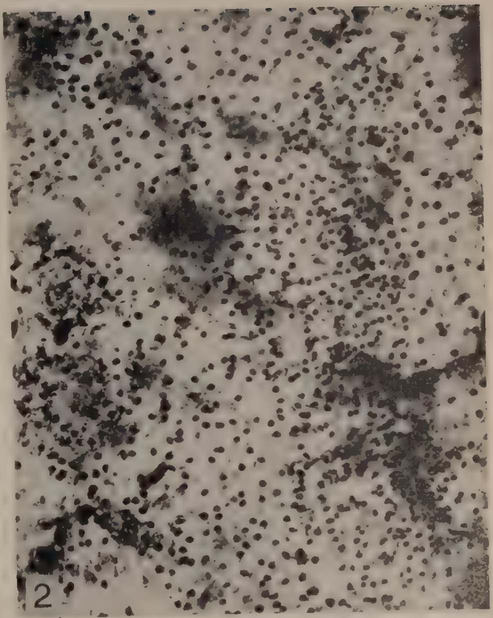
PLATE 1

EXPLANATION OF FIGURES

BRAIN (cortex):

1 Adenosine-triphosphate, pH 9.9. Incubation time: 4 hours. × 80.
2 Adenosine-triphosphate, pH 8.3. Incubation time: 24 hours. × 80.
3 Adenosine-triphosphate, pH 8.3. Incubation time: 24 hours. × 400.
4 Muscle-adenylic acid, pH 9.9. Incubation time: 6 hours. × 80.





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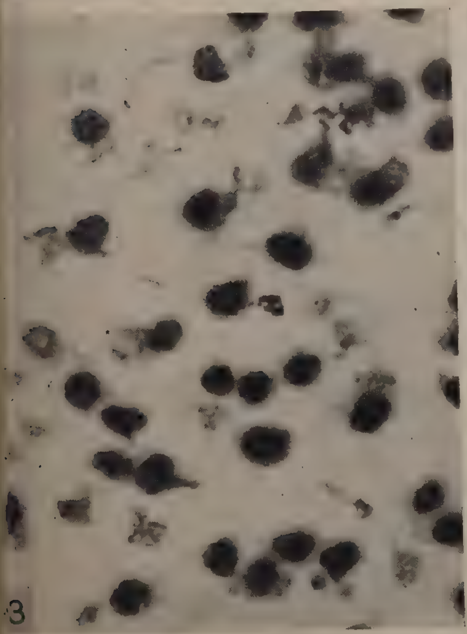

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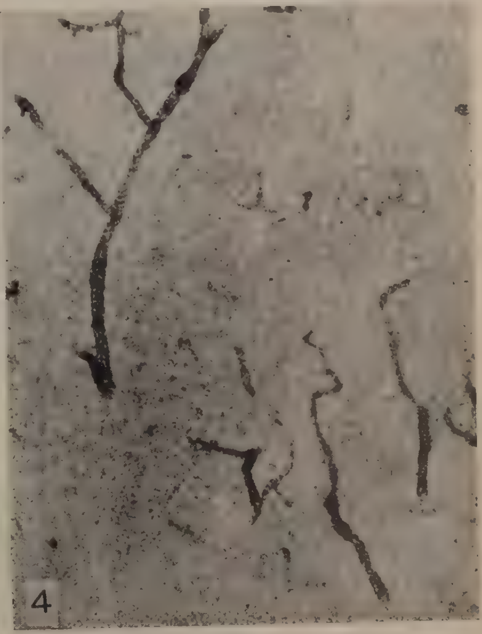

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PLATE 2

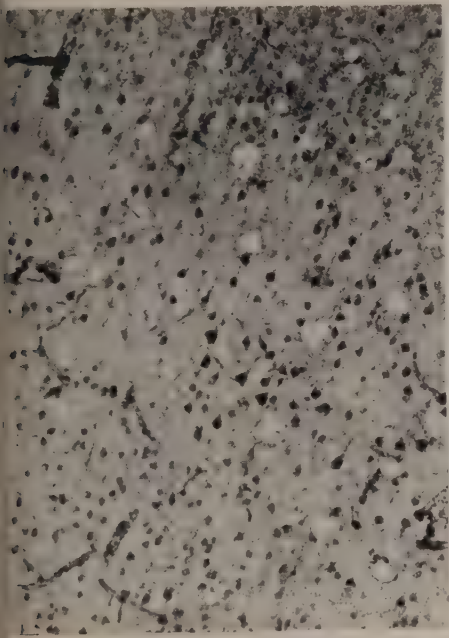
EXPLANATION OF FIGURES

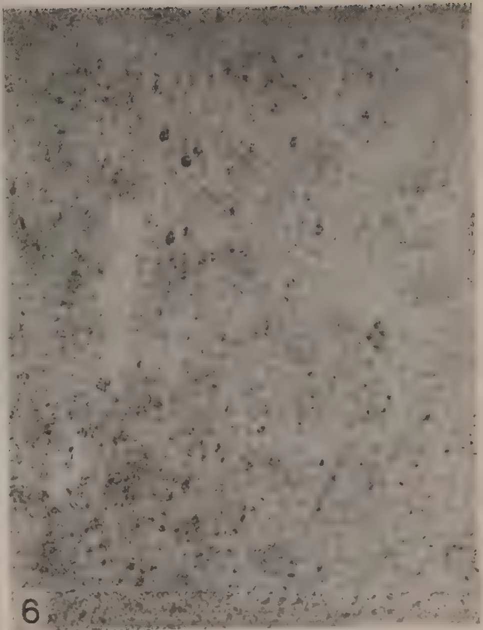
BRAIN (cortex):

5 Yeast-adenylic acid, pH 9.9. Incubation time: 24 hours. × 80.

6 Creatine-phosphate, pH 9.9. Incubation time: 48 hours. × 80.

7 Creatine-phosphate, pH 8.9. Activated with 4.5×10^{-3} M $MgCl_2$. Incubation time: 48 hours. × 80.





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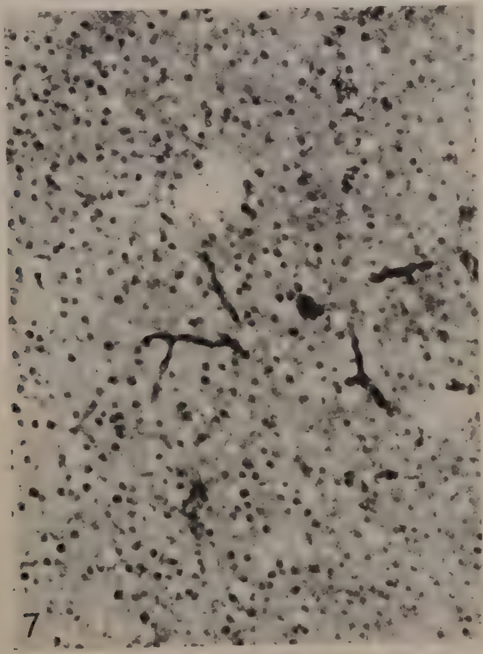

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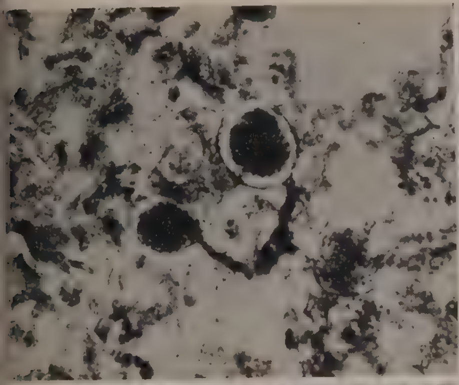
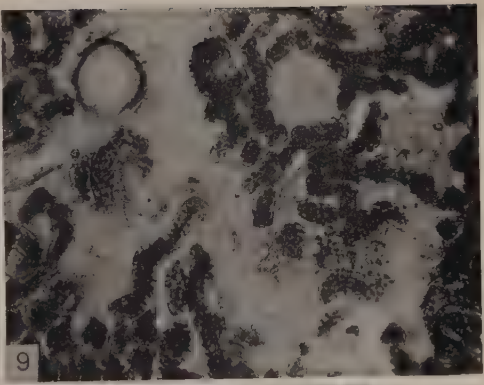
PLATE 3

EXPLANATION OF FIGURES

KIDNEY:

8 Adenosine-triphosphate, pH 9.9. Incubation time: one hour. × 80.
9 Muscle-adenylic acid, pH 9.9. Incubation time: 5 hours. × 80.
10 Creatine-phosphate, pH 9.9. Incubation time: 5 hours. × 80.
11 Creatine-phosphate, pH 8.9. Activated with 4.5×10^{-3} M $MgCl_2$. Incubation time: 22 hours. × 80.
12 Control without substrate. Incubation time: 22 hours. × 80.





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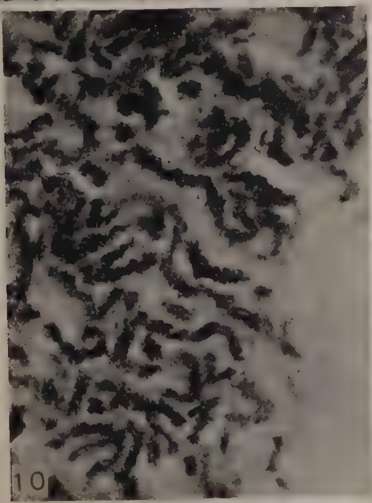

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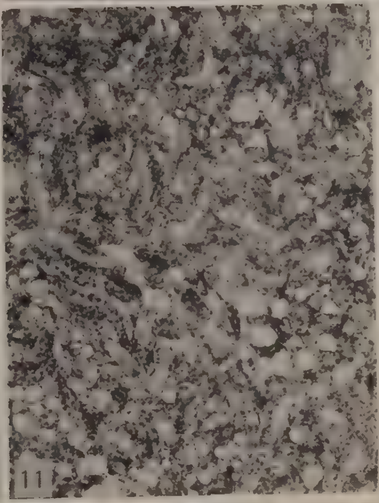

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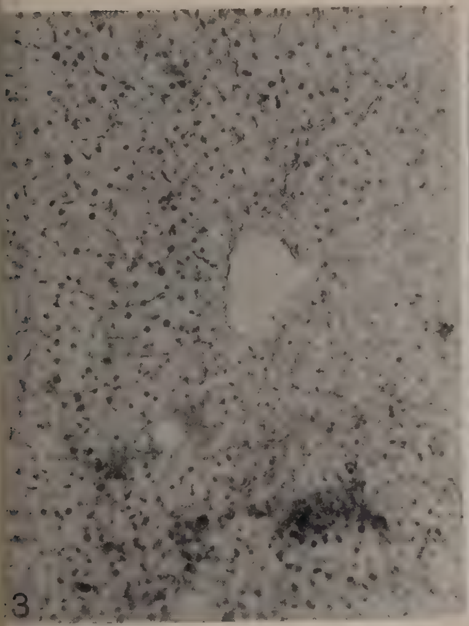
PLATE 4

EXPLANATION OF FIGURES

LIVER:

13 Adenosine-triphosphate, pH 9.9. Incubation time: 4 hours. × 80.
14 Adenosine-triphosphate, pH 8.3. Incubation time: 22 hours. × 80.
15 Muscle-adenylic acid, pH 9.9. Incubation time: 48 hours. × 80.



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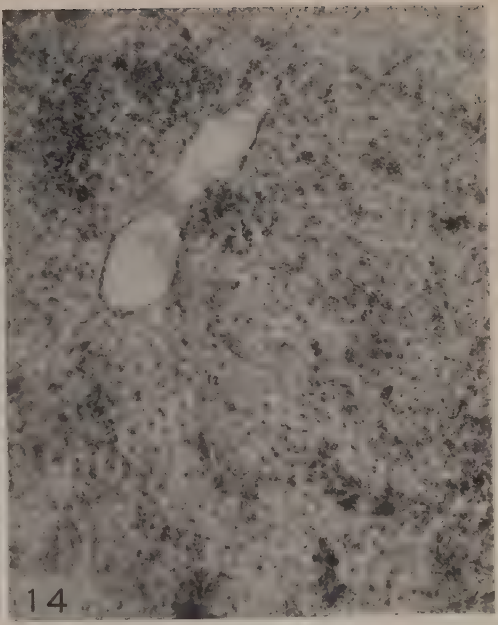

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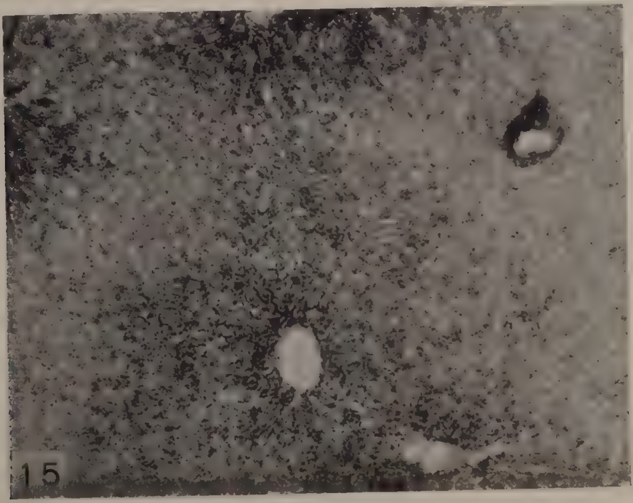

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PLATE 5

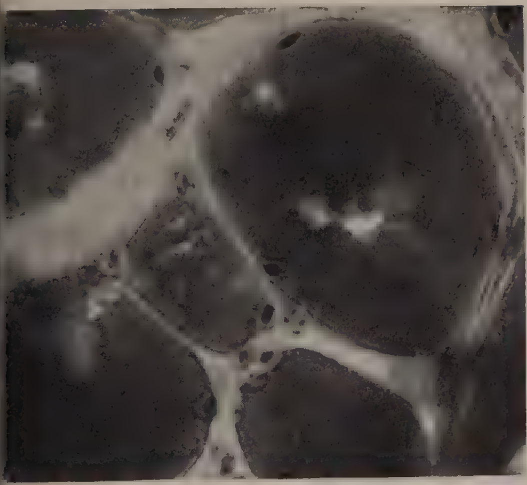
EXPLANATION OF FIGURES

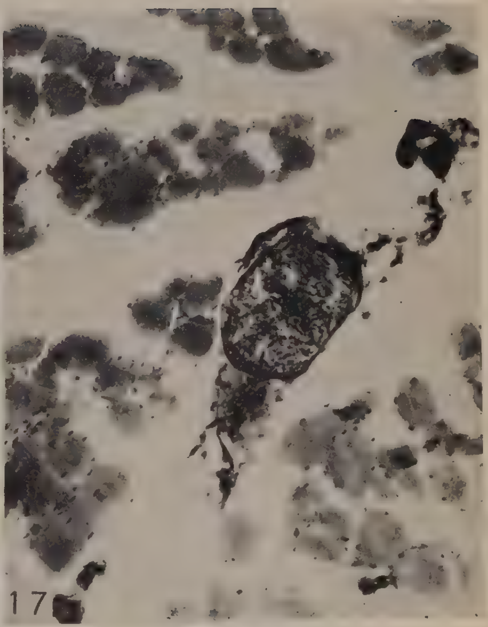
STRIATED MUSCLE (abdominal wall):

16 Adenosine-triphosphate, pH 9.9. Incubation time: 24 hours. × 400.

17 Adenosine-triphosphate, pH 8.3. Incubation time; 22 hours. × 80.

18 Adenosine-triphosphate, pH 8.3. Activated with 2.5×10^{-2} M glutathione. Incubation time: three hours. × 80.





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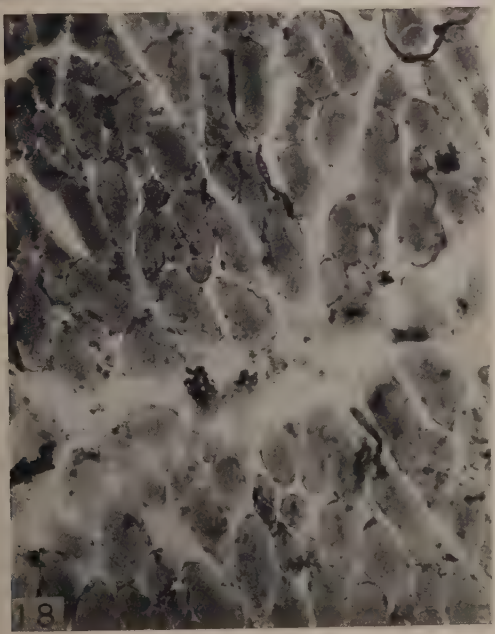

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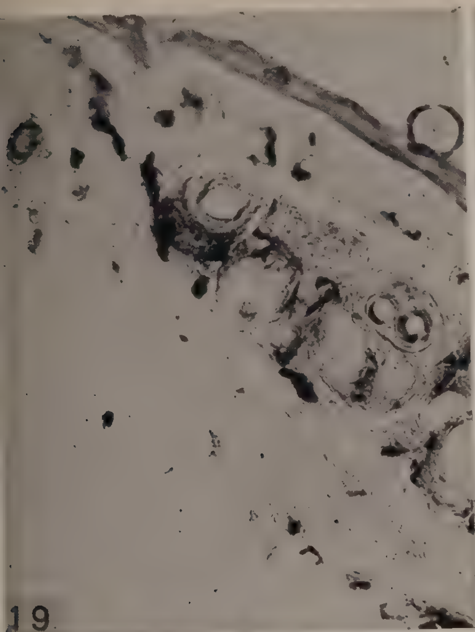
PLATE 6

EXPLANATION OF FIGURES

SKIN (tail):

19 Adenosine-triphosphate, pH 9.9. Incubation time: one hour. × 80.
20 Adenosine-triphosphate, pH 9.9. Incubation time: 4 hours. × 80.
21 Adenosine-triphosphate, pH 9.9. Incubation time: 4 hours. × 400.
22 Control without substrate. Incubation time: 24 hours. × 80.



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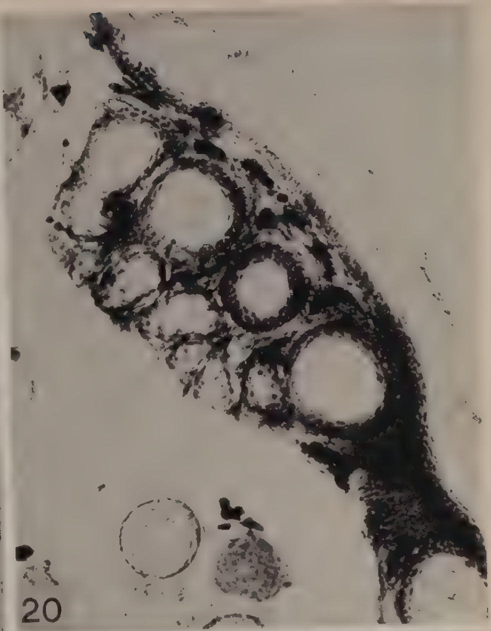

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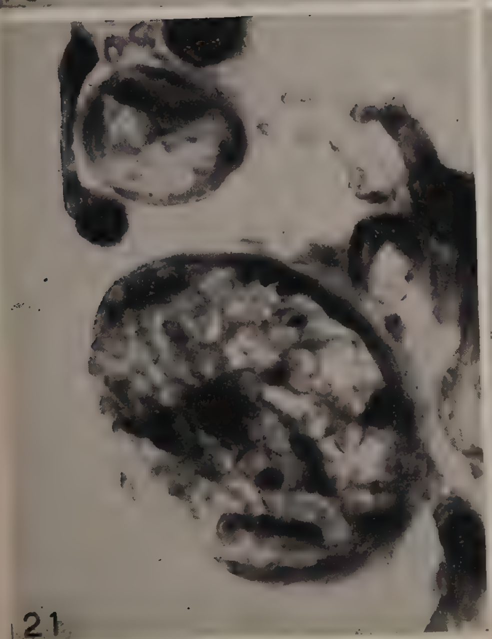

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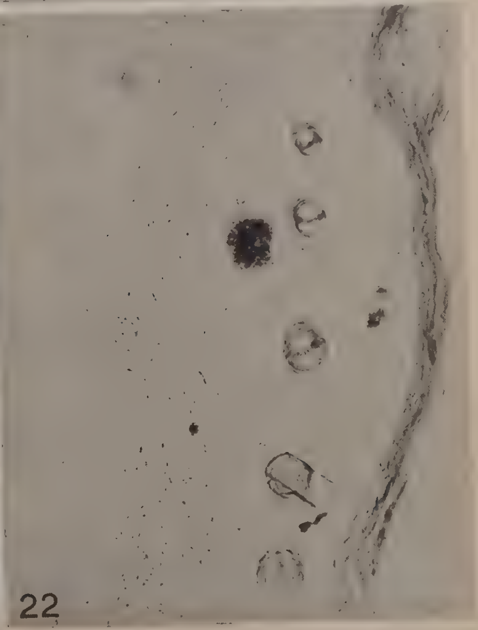

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CHEMOTROPISM IN RHIZOPUS NIGRICANS: THE STALING REACTION[1]

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EIGHT FIGURES

When the irregularly shaped spores of *Rhizopus nigricans* are inoculated onto a nutrient medium in agar, they take up water and swell to spheres of 10 times the original volume before germination takes place. The germ tube which then emerges may appear on any side of the spore, and there is no apparent orientation. But a clump of spores germinating together has a predictable growth pattern. The germ tubes appear, in most cases, on the side directly away from the clump, and they grow out as hyphae away from the group, like spokes of a wheel (fig. 1, above). This oriented growth away from crowded regions toward regions of sparse population results in the mycelium quickly spreading itself over the whole surface of available nutrient, and such a growth pattern may have survival value for those molds possessing it, because it expedites the conversion of what may be a transitory food supply into highly resistant spores.

Earlier studies have demonstrated this tropic growth phenomenon, and it has been called the "staling reaction," as it is essentially orientation away from a region in which the mold has been metabolizing, thus a region of stale medium. The purpose of the present work has been to study the physiology of this growth behavior with two specific objectives: (1) to determine the nature of the stimulus for orientation, and, (2) to learn something of the mechanism of the organism's response to this stimulus.

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[1] The work reported here represents part of the research done toward a Ph.D. degree at Princeton University.

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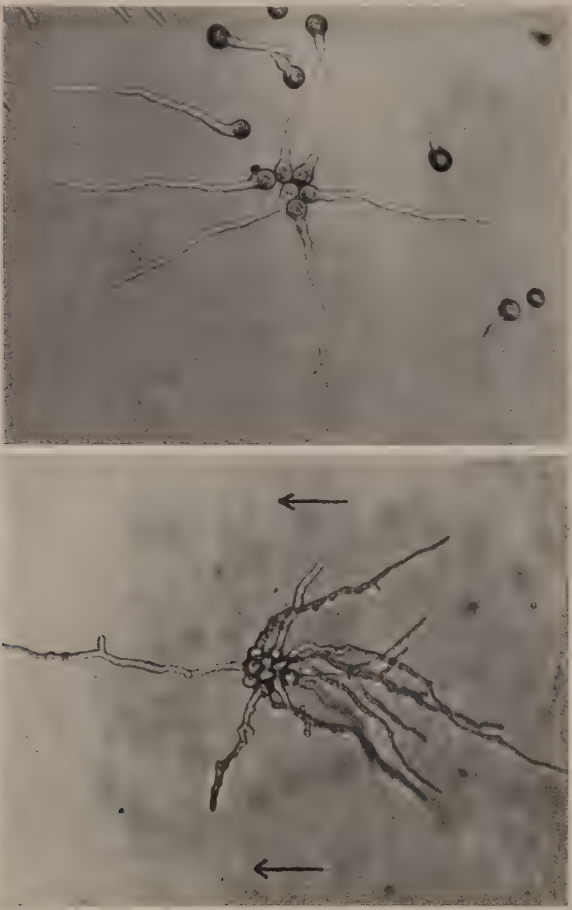

Fig. 1 Above: A clump of germinating spores of Rhizopus showing the staling reaction-growth oriented away from the group. Below: A clump of spores, partially imbedded in agar, which have germinated in a flowing stream of liquid medium. The arrows indicate the direction of flow.

METHODS

For most of the experiments, a method was required whereby the growth direction of spores on the edge of a population could be studied in chemical gradients. For this purpose, plates of Turtox plastic cover slip material about 0.2 mm thick have been used. Four circular holes are drilled in each plate. The holes are spaced well apart and are 1 mm in diameter (fig. 2). A layer of agar medium is placed on each side of the plate so that the two layers are in contact with each other only within the holes. If one layer has been made up with a component which is not in the other layer, then this material will diffuse through the holes into the other layer, and a concentration gradient will be set up in the regions in and near the holes. The direction of growth of spores in these regions can then be studied. This is a modification of the method of Miyoshi (1894) and later workers who used mica plates with pin holes. The present method has the advantage that the holes are perfect circles rather than the jagged incisions made by perforating mica. This greatly simplifies the geometry of gradients arising from diffusion through the hole.

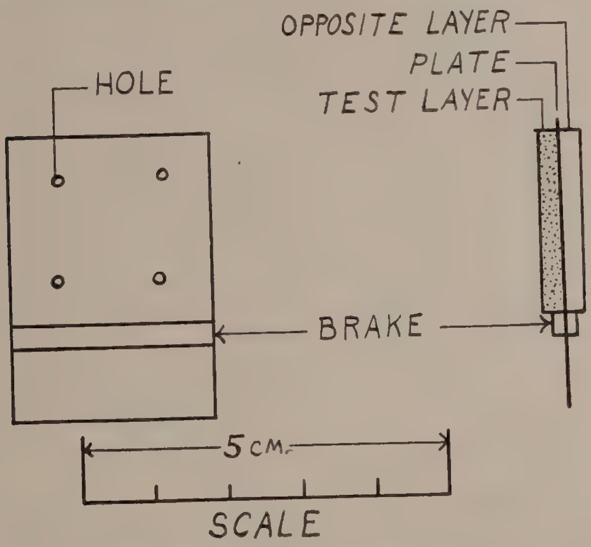

Fig. 2 Front and side views of one of the test plates. It is in the vertical position during incubation. The brakes prevent slipping of the agar layers.

The material is prepared by mixing 4 ml of liquid medium with 2 ml of melted 6% agar. This is cooled to 40°, and a 2 ml aliquot is poured on a glass slide. The plastic plate is carefully lowered onto the agar and held in position until the agar solidifies. Then the plastic plate and agar layer are slipped off the slide together, inverted, and lowered onto cooling agar on another slide.

If spores are to be mixed into an agar layer, then only 3 ml of liquid medium is originally mixed with the 2 ml of 6% agar. After this has cooled to 40°, and just before pouring, 1 ml of spore suspension is added. The spores are suspended in water, and concentration is determined by counts in a haemocytometer.

The experiments here reported, unless otherwise specified, have employed a synthetic nutrient medium containing 1% glucose, 2×10^{-3} molar $MgSO_4$, 1.1×10^{-2} molar KH_2PO_4, 7.6×10^{-3} molar asparagine, and 2% agar. The stock slants also contained this medium.

The agar layer containing the spores of which the growth direction is to be studied is called the "test layer." The other layer is designated as the "opposite layer." The opposite layer may or may not contain spores, depending on the experiment.

Observations are made only on those spores in the test layer which are in the region immediately around one of the holes. This is called the "test region." It is the shape of a metal washer, with an inner diameter of 1 mm (the diameter of the hole), an outer diameter of 2 mm, and a depth of 0.1 mm. Thus only those spores near the hole and very close to the surface of the plate are studied.

The preparations are incubated in a moist chamber at 28° for 8 hours and 30 minutes. By this time most of the hyphae have reached a length of 10 to 20 spore diameters. They are then fixed and stained with Amman's cotton blue. A camera lucida drawing (× 80) is made of each test region, and the growth direction for each spore is measured with a protractor (fig. 3). The measurement is of that

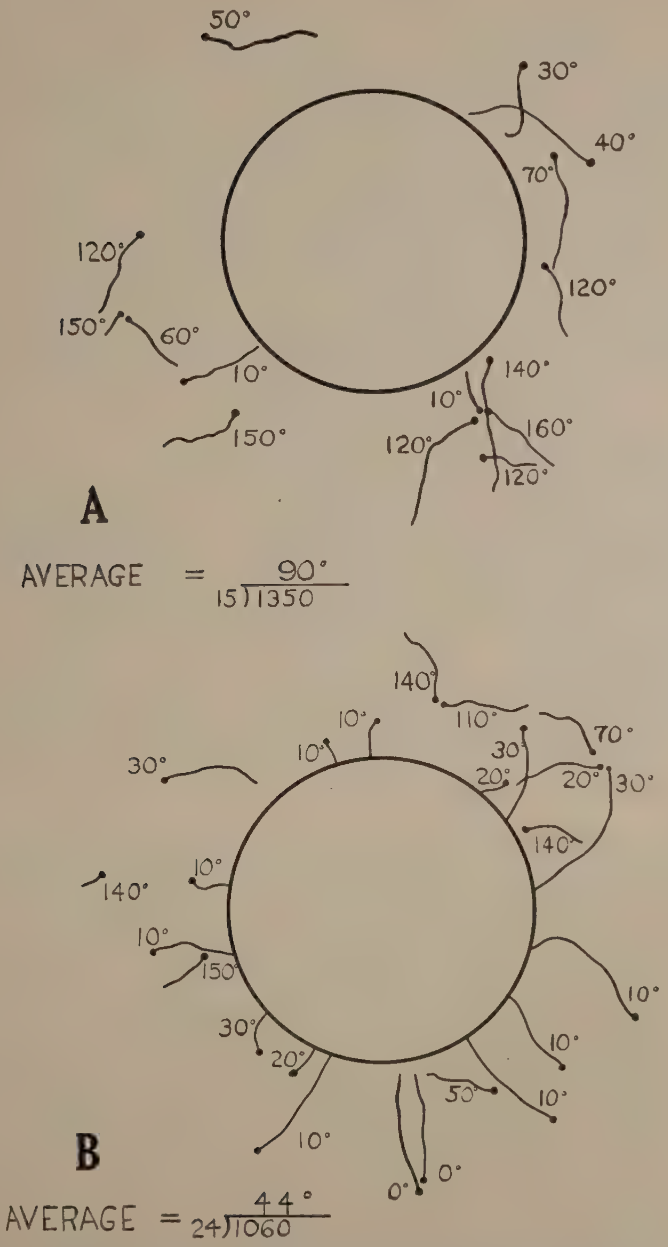

Fig. 3 A: Camera lucida drawing of a test region on a plate with spores in synthetic medium in the test layer and also in the opposite layer. B: Camera lucida drawing of a test region from a plate with spores in synthetic medium in the test layer and non-spore synthetic medium in the opposite layer.

angle of which the spore is the vertex, one arm is the line to the tip of the hypha, and the other arm is the direction toward the center of the hole (this is the direction of any chemical gradient set up between the two layers). The angles are measured to the nearest 10°. If the growth directions are random, than all the spores in a test region should average out to around 90°, since the limiting values are zero and 180°. If there is orientation toward the hole, the average angle becomes less than 90°. This method of measurement has been chosen because the angles can be quickly and accurately determined. The method is empirically justified in that it consistently gives results which conform to the experimental hypothesis. That is, it gives averages close to 90° in experiments prepared so that there is no gradient in the test regions, while it gives results significantly different from randomness when certain kinds of gradients are imposed. It is possible that a more discriminating method of measurement could be devised which would detect a change of orientation where this method showed none, but for these exploratory experiments it was felt that speed should be stressed so that a great many observations could be made.

THE STALING REACTION

If an experiment is prepared with spores in the nutrient medium in the test layer and the same concentration of spores in the same medium in the opposite layer, then the system is everywhere homogeneous, and no gradient can be set up. The result is random growth (fig. 3 a). But if the test is done with spores in nutrient medium in the test layer and the same medium with no spores in the opposite layer, then we find that the growth in the test regions is oriented toward the holes (fig. 3 b). The average angles are 40–45° (table 1). This is the staling reaction again, growth away from the crowd into a region of more sparse population.

Assuming, for the moment, that this oriented growth is a response to a chemical stimulus, it immediately becomes apparent that there are two sorts of chemical gradients which could be responsible. The spores, in their metabolism, have used up some of the nutrient materials in the test layer, so there will be increasing amounts of these in the direction through the hole into the other layer. If the growing tip were sensitive to the concentration of sugar or some other nutrient, then the orientation could be a response to the gradient of this factor. On the other hand, any products of the mold metabolism are more concentrated in the test layer than the opposite layer, and the orientation could be tropic growth away from one of these.

TABLE 1

TEST LAYER: 90/mm^3 spores in synthetic medium. OPPOSITE LAYER: 90/mm^3 spores in synthetic medium.	TEST LAYER: 90/mm^3 spores in synthetic medium. OPPOSITE LAYER: non-spore synthetic medium.	TEST LAYER: 90/mm^3 spores in synthetic medium. OPPOSITE LAYER: non-spore water agar.
81 degrees	38 degrees	44 degrees
99 degrees	41 degrees	41 degrees
95 degrees	48 degrees	42 degrees
93 degrees	56 degrees	49 degrees
Mean: 92 degrees	Mean: 46 degrees	Mean: 44 degrees

Each figure given in the tables is the average angle for the spores in the test region of one hole.

The 92 degree mean is significantly different from the 46 degree mean at the 1% level. $t = 8.26$ with 6 degrees of freedom.

A simple experiment shows which is the correct explanation. A plate is prepared with the test layer again containing spores in nutrient medium, while the opposite layer is made up with no spores and no nutrients, only water agar. When the test regions are examined it is found that there is again orientation toward the hole with the same angle of 40–45° (table 1). In this case, they could not be growing toward increasing levels of any nutrient, since there were no nutrients in the opposite layer.[3] This experiment was first performed by Clark ('02), who concluded from it that the orientation was a negative chemotropic response to some product of the mold's metabolism. He thus corrected the mistaken interpretation of Miyoshi (1894), who had believed that the germ tubes grew toward the hole because they were attracted[4] by the nutrients he had placed on the other side.

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[3] Another possibility is that there is some factor in the *agar* which is being used up in the spore layer and that the tropic growth is a response to the gradient of this material. That the staling reaction is not dependent on such a factor is shown by cultures in liquid medium, where spores in clumps are seen to grow away from the group just as in agar.

NATURE OF THE STIMULUS: CHEMICAL OR PHYSICAL?

Studies with cell-free staled media. The orientation is evoked by some material or condition of the medium which is manifested by the presence of the living mold. It now becomes desirable to know something more of the nature of this tropic stimulus. Is it a chemical product of the mold's metabolism, or is it some physical condition of the medium caused by the presence of the mold? If it is chemical material, diffusing into the medium from the metabolizing Rhizopus, then its presence in the medium should be demonstrable even after its source (the mold) is removed. Spores inoculated near such a cell-free, staled medium should orient their growth away from it. Such a result has never been achieved in this study. Media in which rich cultures of Rhizopus had grown were filtered to remove the mold and then used as opposite layer material in tests on the tropism plates. The spores in the test regions of these plates grew toward the holes just as pronouncedly as on those plates with fresh medium in the opposite layer. This experiment was done repeatedly with the same result.

It was considered that the staling factor might be chemical material of a labile character such that it was present in active form in the staled opposite layer at the start of the experiment but disappeared before the end of the 8½ hour incubation period. In view of this possibility, tests were done in which the filtered, staled medium of the opposite layer was replaced every one and one-half hours throughout the incubation period with a newly filtered preparation. Still the test spores oriented just as markedly toward the hole (table 2). On the chance that the staling factor was being actively broken down by an enzymatic reaction, the staled medium was heated to 100° immediately after filtration, but the medium still failed to demonstrate active staling factor (table 2). It is concluded from these experiments that the stimulus for the staling tropism is something that disappears from a medium very quickly after its source, the growing mold, is removed.

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[4] The term "attraction" is used in this paper as a label for positive tropic action; "repulsion" is used to refer to negative tropic action. It is recognized that true attraction involves a physical force exerted by that which attracts on that which is attracted. We do not assume that such forces are involved in the phenomena discussed here, and the terms are used only for convenience.

TABLE 2

TEST LAYER: $80/\text{mm}^3$ spores in synthetic medium. OPPOSITE LAYER: non-spore synthetic medium.	TEST LAYER: $80/\text{mm}^3$ spores in synthetic medium. OPPOSITE LAYER: non-spore, 12 hour staled synthetic medium.	TEST LAYER: $80/\text{mm}^3$ spores in synthetic medium. OPPOSITE LAYER: non-spore, 12 hour staled medium, boiled one minute immediately after filtration.
63 degrees	39 degrees	56 degrees
38 degrees	43 degrees	42 degrees
49 degrees	45 degrees	40 degrees
46 degrees	55 degrees	44 degrees
Mean: 49 degrees	Mean: 45.5 degrees	Mean: 45.5 degrees

Opposite layers in all these tests were replaced every 1½ hours.

An early attempt to demonstrate the staling factor in cell-free, staled medium was made by Fulton ('06). Working with 8 different molds, he observed slightly less orientation toward staled medium than toward fresh, but his results were not conclusive. Later, Graves ('16) studied the tropic behavior of Rhizopus spores in turnip juice medium and observed what he believed to be a strong staling reaction, elicited by staled medium. When he put spores in turnip juice in the test layers of his plates and fresh turnip juice with no spores in the opposite layers, the spores grew toward the holes very markedly. If the opposite layer contained filtered, staled turnip juice, then the spores in the test region grew in random directions or slightly *away* from the holes. He believed that this staled medium was showing the presence of the stimulus for the staling tropism, and therefore he concluded that the stimulus was chemical material produced by the metabolizing mold. Graves called this material the "staling substance."

Since the results of the experiments in the present study on cell-free, staled media are directly contradictory to those of Graves, it was deemed advisable to repeat his experiments. The essential difference between the two methods is that Graves used turnip juice media while the current experiments have been done in synthetic media. The results of repeating the experiments in turnip juice largely confirm those of Graves, but a comparison of these results with those in synthetic media suggests an interpretation different from his.

TABLE 3

TEST LAYER: 60/mm^3 spores in synthetic medium. OPPOSITE LAYER: non-spore fresh turnip juice.	TEST LAYER: 60/mm^3 spores in fresh turnip juice. OPPOSITE LAYER: non-spore fresh turnip juice.
14 degrees	24 degrees
11 degrees	23 degrees
24 degrees	19 degrees
17 degrees	26 degrees
Mean: 16.5 degrees	Mean: 23 degrees

Turnip juice contains something which exerts what appears to be a powerful attraction [5] effect on Rhizopus spores in these tropism tests. When spores in synthetic medium are grown on a plate opposite non-spore turnip juice, they show extreme orientation toward the turnip juice with an average angle of only 15–20° (table 3). Even if they are *in* turnip juice opposite non-spore turnip juice, they show markedly better orientation than the 40–45° average of the staling reaction alone (table 3); apparently the "attractant" is being used up in the spore layer, setting up a rising concentration gradient of it in the direction through the hole into the opposite layer. Graves noted the "attraction" affected by turnip juice but did not recognize its part in his staled medium experiments.

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[5] This material does not exercise a true tropic action on Rhizopus, and the nature of its function will be discussed elsewhere.

When spores in turnip juice were grown opposite filtered, staled turnip juice in this study, they grew very slightly toward the hole, on the average, with an angle of 71° (table 4). When the experiment was done with the same test layer (spores in turnip juice), but with *fresh synthetic* medium in the opposite layer, the very comparable average of 76° resulted (table 4). We know that this fresh synthetic medium contained no staling substance and no "attractant." It elicited the same growth behavior as turnip juice in which mold had grown. What had changed about the turnip juice content in this treatment to convert it from a powerful "attractant" to this ineffectual state? It could have built up enough staling factor to completely neutralize the "attraction." Or it could have used up or destroyed its "attractant" without any buildup of staling substance concentration. We know that in synthetic medium, which contains no "attractant" (and therefore cannot cause this ambiguity), the cell-free, staled medium fails to show any stored-up staling substance. We know from the experiment with spores in turnip juice opposite fresh turnip juice that the presence of spores seems to use up the "attractant" in their immediate surroundings.

TABLE 4

TEST LAYER: 60/mm³ spores in fresh turnip juice. OPPOSITE LAYER: non-spore, 25 day staled turnip juice.	TEST LAYER: 60/mm³ spores in fresh turnip juice. OPPOSITE LAYER: non-spore synthetic medium.
67 degrees	62 degrees
85 degrees	77 degrees
66 degrees	70 degrees
67 degrees	94 degrees
Mean: 71 degrees	Mean: 76 degrees

Graves reported actual turning *away* from staled turnip juice. My results show very significant difference from orientation toward fresh turnip juice, but still slight turn toward the hole.

Therefore, the most plausible conclusion appears to be that the staled turnip juice is not showing the presence of a repellant (staling substance), but rather, the absence of an "attractant."[6]

Experiments on free-diffusing factors. The failure of cell-free, staled medium to show the presence of active staling factor leads to doubt as to whether this factor is actually chemical in nature. It could be either some very labile chemical substance or some physical factor which sets up a gradient around the growing mold. For example, the hyphae might all elicit the same electric charge in their immediate surroundings, and electrical repulsion could be effective in determining the position of new protoplasm being laid down at the hyphal tips.

Two experiments were designed to determine whether the stimulus for the staling reaction was a freely diffusible factor. In the first, tropism plates were prepared with the test layers made up as usual with spores in synthetic medium. The opposite layer was put on in the usual fashion, but then, in the region around each hole, a circular disk with a diameter 6 times that of the hole was cut out of the opposite layer. This space was filled with non-spore synthetic medium. The rest of the opposite layer, on the experimental plate, contained a heavy inoculation of spores in synthetic medium (non-spore medium on the control). The spores in the opposite layer were all at least 2½ mm from the hole. Even so, they had a profound influence on the growth direction of the spores in the test layer (table 5). Thus, the staling factor showed its effect at least 150 spore diameters from its source.

Another experiment which substantiated the chemical hypothesis was one in which spores were allowed to germinate in a flowing stream of liquid medium. A glass slide was covered with a thick layer of agar with a flat-bottomed channel running down the middle (this was made with a plastic mold). Spores were inoculated onto the agar floor of the channel while it was still liquid, so that they were anchored when the agar solidified. A constant flow (50 ml/hour) of liquid medium passed down the channel throughout the incubation period. When the culture was examined, it was found that hyphae from clumps of spores nearly all turned and grew upstream (fig. 1, below). This seems to indicate that the center of staling factor concentration was shifted a little downstream from the center of the clump (fig. 4). If the staling factor was carried by the stream, washed along from the spores where it was produced, then it was certainly behaving like a chemical substance.[7]

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[6] Graves found further that boiling the staled medium removed much of its repulsive effect. He thus concluded that the staling substance was highly volatile. I have been unable to confirm this finding. Boiling had little or no effect on the tropic action of my staled turnip juice.

TABLE 5

TEST LAYER: 70/mm^3 spores in synthetic medium. OPPOSITE LAYER: non-spore synthetic medium.	TEST LAYER: 70/mm^3 spores in synthetic medium. OPPOSITE LAYER: 200/mm^3 spores in synthetic medium.
39 degrees	73 degrees
26 degrees	79 degrees
37 degrees	54 degrees
71 degrees	79 degrees
Mean: 43 degrees	73 degrees
	74 degrees
	79 degrees
	92 degrees
	Mean: 75 degrees

Disk-shaped regions immediately around holes in opposite layers were non-spore synthetic medium on all plates.

The 43 degree mean is significantly different from the 75 degree mean at the 1% level. t = 4 with 10 degrees of freedom.

Unique among the common physical phenomena, heat diffuses out through a chemical environment in the same manner as chemical diffusion. Although the foregoing experiments would seem to rule out such physical forces as light and electricity as the essential nature of the staling stimulus, it

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[7] It has been suggested that the turning upstream might be oriented growth against the mechanical force of the stream, thus negative rheotropism. This is very unlikely, for if the upstream growth were mechanically evoked, the lone spores should grow that way as regularly as those in clumps, and they do not.

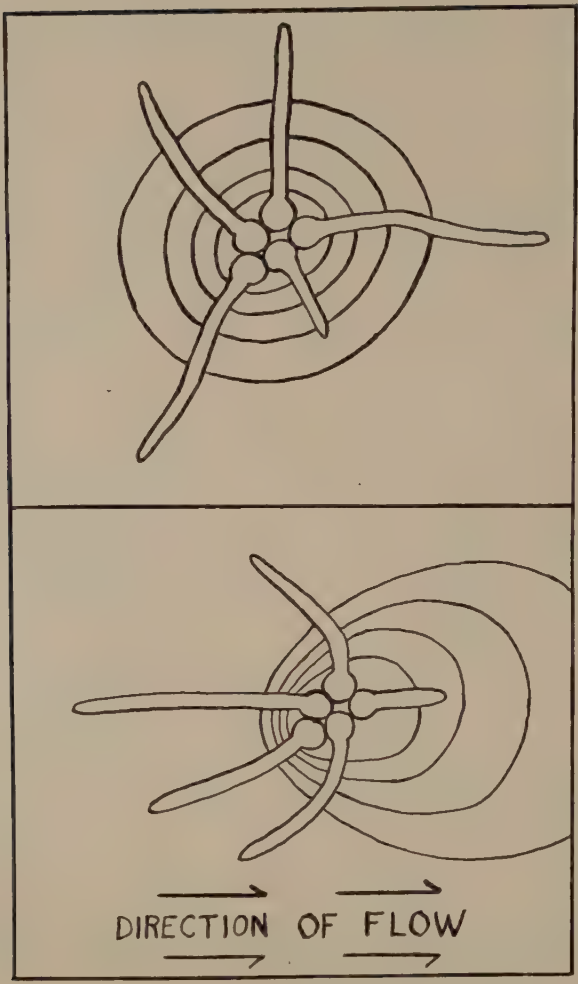

Fig. 4 "Contour maps" of staling substance concentrations around clumps of germinating spores. This is a hypothetical interpretation of the experimental finding that the hyphae of a clump germinating in flowing, liquid medium turn upstream. The upper drawing shows a clump germinating in a stationary environment. The concentration of staling substance diminishes uniformly all around the clump. Hyphae grow straight out toward lower concentrations, thus perpendicular to the contour lines of constant concentration. If the clump is in a stream, as in the picture below, it is postulated that the center of staling substance concentration in the agar near the surface is displaced downstream, and the hyphae, again growing perpendicular to the contour lines, turns upstream.

is still possible that heat gradients cause the staling reaction. An experiment has been performed which shows that this is not the case. Two brass chambers with flat, rectangular tops were built into a box so that they are separated by a gap 5 mm wide. The temperature of the brass plates was maintained by water circulating through the chambers from constant temperature baths.[8] The baths were kept at 36° and 13°.

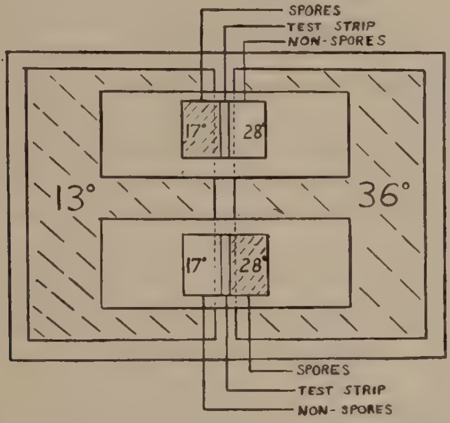

Fig. 5 The heat gradient experiment as seen from above. The glass slides holding the agar preparations are placed across the gap between the two constant-temperature brass plates.

A pair of glass slides were prepared with narrow (2 mm wide) strips of agar containing synthetic medium and a low concentration of spores. This was flanked, in each case, by non-spore agar on one side and a heavy culture of Rhizopus on the other. In the absence of other heterogeneity, the hyphae in the center strip would turn toward the non-spore side. The slides were placed on the temperature gradient box with the test strip directly over the gap between the plates and with the non-spore agar on the hot side in one case and the cold side in the other (fig. 5). The temperature in the agar came to 28° on the hot side and 17° on the cold side. On both plates the spores in the test strip grew toward the non-spore side.

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[8] The apparatus for temperature gradients was kindly lent by Dr. C. S. Pittendrigh.

The 11° drop in temperature was accomplished in a distance of less than a centimeter, so the gradient was certainly steep enough to overcome any heat gradient set up by the mold itself. Since the tropic reaction we are studying took place in the face of a temperature gradient in both directions, this is strong evidence against heat being the stimulus for this reaction. All the evidence points to the staling factor being an unstable chemical substance.[9]

SOME CHARACTERISTICS OF THE STALING REACTION

When a growing spore layer opposite a non-spore layer orients toward the hole in the plate, this orientation is not all a result of hyphal turning. The germination (direction of emergence of the hypha) shows orientation too. If we measure the germination angles (angle between direction of emergence and direction toward center of hole) of the test region of such a preparation, we find they average about 55–60°. The final angle of hyphal growth averages 40–45°. This means that the hyphae turn 15°, on the average, toward the hole.

There is evidence that staling substance production increases as the mold grows. Some must be produced by the pre-germination spores to account for the orientation in germination (assuming the same substance is responsible for the orientation in germination and in hyphal growth). The fact that the staling substance level increases is shown by experiments in which the opposite layer is like the test layer in spore concentration but different in age. If the opposite layer is allowed to develop 6 hours before the experiment starts, the result is pronounced turning away from the hole by spores in the test region. If the opposite layer is kept "young" throughout the experiment by periodic replacement with a freshly mixed culture, then growth in the test region turns toward the hole almost as readily as toward a non-spore layer.

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[9] There is no reason for believing, at present, that the stimulus for the staling reaction is a single substance rather than a group of substances, but the term staling substance will be used here for convenience.

It is possible also to demonstrate that staling substance level changes with spore concentration. If the opposite layer contains spores in a concentration twice as high as the test layer, the spores in the test region grow away from the hole. If the spore level in the opposite layer is one-half of what it is in the test layer, then the test spores grow toward the hole, though not as pronouncedly as toward a non-spore opposite layer. Similarly, in a series of tests with various spore concentrations in the test layers (with non-spore opposite layers), the orientation improves (average angle decreases) with increasing population density.

MECHANISM OF TURNING IN THE STALING REACTION

The growth factor hypothesis. It is known that tropic turning in higher plants is mediated through unequal distribution of the growth substance auxin, and that the side with more auxin grows faster (in shoots), resulting in a turn toward the other side. This is substantiated experimentally by growing shoot pieces in homogeneous auxin solutions of various concentrations and showing that the more concentrated the auxin, the faster it grows.

It was felt that tropic turns in Rhizopus might be the result of differential growth rate on the two sides of the hypha resulting from heterogeneous distribution of a growth factor or factors in its surroundings. If the growing mold produced a growth factor which was in limiting concentration and released it into the medium, then it would be most concentrated in regions of heavy population. A hypha growing tangent to such a mass would be in a higher concentration of growth factor on the side toward the heavy culture than on the side away. If opposite sides of the hypha could grow at rates independent of each other, then this hypha would grow more on the side toward the thick culture and would turn away from it. Thus we have a hypothetical scheme for a limiting growth factor as the staling substance.

The first test of this hypothesis is to determine whether or not the medium in a highly populated region actually does enhance growth rate. This experiment is easily done by measuring linear growth rate of individual hyphae in sparse cultures and in heavy cultures. The spores are allowed to germinate in a layer on the under side of a cover slip over a Van Tieghem ring. Camera lucida drawings are made of the same spores at intervals of about one hour, and length of hypha is plotted against age. If the hypothesis is valid, growth must be faster in the heavy culture, which it is. A 25-fold increase in spore concentration (6/mm^3 to 150/mm^3) gives growth rates two to three times as fast in hyphae over 100 μ long (fig. 6). (Germination time and very early growth do not seem to be affected by the concentration of spores. The tropic tests on the plates run until the hyphae are mostly 300–500 μ long.)

The result of this experiment shows that the growing mold is producing a limiting growth factor or factors which diffuse into the medium. This fits the hypothesis that the staling reaction is the result of differential growth mediated by a factor produced in the mold. But another experiment fails to substantiate this view. Synthetic media were inoculated with heavy spore concentrations of Rhizopus and incubated 11 hours in one case and 48 hours in another. The spores and hyphae were removed by filtration and tropism tests were run using these staled media for the opposite layers. The result was negative, that is, the hyphae oriented toward the hole as markedly as if fresh medium were on the other side. At the same time, growth rate studies were made on a given spore concentration in fresh medium and in these two staled media. Both staled media caused marked acceleration of growth. Thus growth factors from the staled medium remain when the staling substance has disappeared.

Another difficulty with the staling substance growth factor hypothesis is that it does not seem to be one which could account for oriented *germination.* (Although we are not sure that the same factor controls germination direction and hyphal turning, we have never found a situation in which one functioned without the other, so it would be a reasonable assumption to consider them related processes.) The germ tube emerges on the side *away* from concentrated staling substance. It would seem that the role of the staling substance must be one of growth suppression rather than facilitation, in this regard.

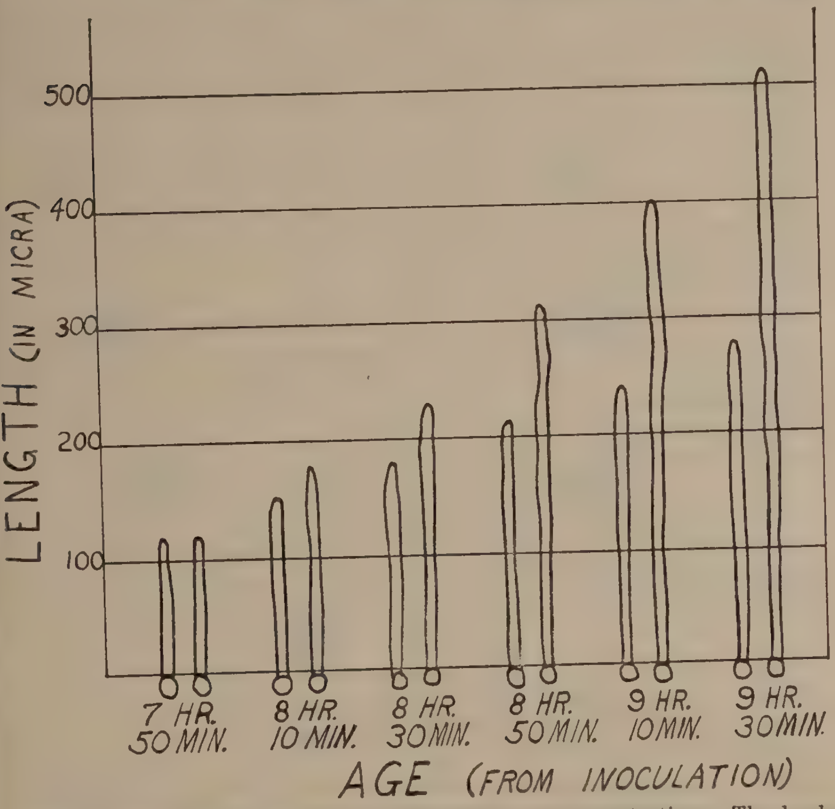

Fig. 6 Rate of hyphal growth at different spore concentrations. The hypha on the left in each pair represents the average length of 10 hyphae growing in synthetic medium with a spore concentration of 6/mm^3. The righthand member of each pair shows the average length of 10 hyphae growing in 150/mm^3 spores.

Also, the region of elongation in these hyphae is different from higher plants. In the latter case, there is a non-growing tip, and elongation takes place in a "collar" a short way back. In such a situation it is clear that faster growth on one side would cause a turn toward the other side. But in the young hyphae of Rhizopus, growth seems to be confined to the very tip, as has been shown by observations in a suspension of carbon particles (fig. 7), and there is no demonstrable elongation farther back. It is as though growth were not a process of elongation of the sides, but a continual opening of the tip and a pouring forth of new material through this apex. Surely tropic turning through differential growth is difficult to envisage in this system.

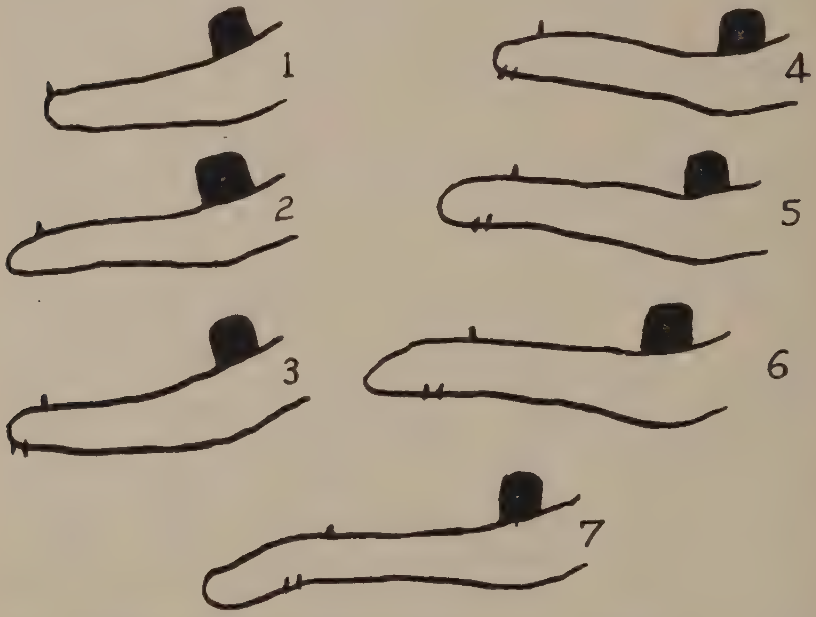

Fig. 7 Camera lucida drawings made at one minute intervals of a hyphal tip growing in a water suspension of carbon particles. The unchanging spatial relationship of particles stuck on the surface indicates that growth takes place only at the very tip.

The wall strengthener hypothesis. A second hypothesis for the mechanism of turning will now be offered which seems more competent to fulfill the requirements of orientation both in germination and hyphal growth. When a spore is placed in liquid medium, it immediately begins to take up water. Before germination, the spore swells to 10 times its original volume by osmotic uptake of water. By this time there must be a strong pressure on the walls. Let us assume that there is an incessant osmotic pressure in spores and hyphae, a pressure to grow, and that the limiting factor is the loosening or weakening of a point in the wall to allow enlargement. Growth, then, would be a "bulging forth" of compressed protoplasm at the weakest point in the wall (just as a balloon bulges at its weakest point when inflated). The staling substance would be a kind of wall strengthener, something which prevented walls in its presence from becoming soft or weak. Thus spores would only be able to emit a germ tube on the side away from a clump since the side toward the clump would be so fortified with staling substance. The same process could control tropic turning at the growing tip of the hypha, if growth here were actually confined to bulging forth from the very tip, as seems to be the case.

Experiments have been started to test this "wall strengthener" or "growth suppressor" hypothesis of staling substance action. If high staling substance concentration on the side of a spore toward a clump prevents germination on that side, then a spore with large amounts of staling substance all around it should be unable to germinate at all. Spores were inoculated onto the surface of a nutrient agar. After a few hours, when these spores had grown short germ tubes, a second inoculum of spores was sown among them. Since staling substance concentration increases with age, there is a large amount of it from the first inoculum surrounding the spores of the second inoculum. These young spores fail to germinate in many cases, but the result is not clear-cut. Sometimes they germinate as well as the control spores, which are in the presence of no previously growing mold. It is felt that this is because the test spores are getting not only staling substance from the previous inoculum but growth factors as well. Therefore, an attempt has been made to design the experiment in a way that removes this ambiguity and leaves staling substance concentration as the only variable between the experimental and the control media. This has been possible because the growth factors produced by Rhizopus (some of them, at least) are stable, as shown by the earlier growth rate experiments, while the staling substance is very unstable.

The experiment was done with liquid medium flowing over partially imbedded spores and was carried out in molded agar channels like the ones described in an earlier section. Sixteen hundred milliliters of liquid synthetic medium was inoculated with Rhizopus and incubated for two days. At this time the mycelium was filtered off, and the filtered medium was allowed to stand 3½ hours before the start of the experiment. It was then placed in a large flask fitted with two capillary siphons. The mycelium was retained in a small amount of medium and kept for the experiment.

Two water-agar channels were prepared with spores partially embedded in the channel floors. They were incubated at 23° in a flowing stream (50 ml/hour) of the used liquid medium. The medium flowing across the experimental preparation passed from one of the capillary siphons onto the old mycelium, which was contained in a filter-lined funnel. The medium, after washing across the mycelium, passed immediately into the channel and over the spores (fig. 8). The control preparation got its medium directly from the capillary siphon. Thus both preparations were getting medium containing the stable products of Rhizopus metabolism (growth factors), but only the experimental was getting the unstable products (staling substance and possible unstable growth factors). After 10 hours' incubation the preparations were fixed and stained. The experimental culture showed only 10–15% germination, while the control had 35–40% germination and markedly better growth. This demonstrates that the unstable products of Rhizopus metabolism suppress, rather than accelerate, growth.

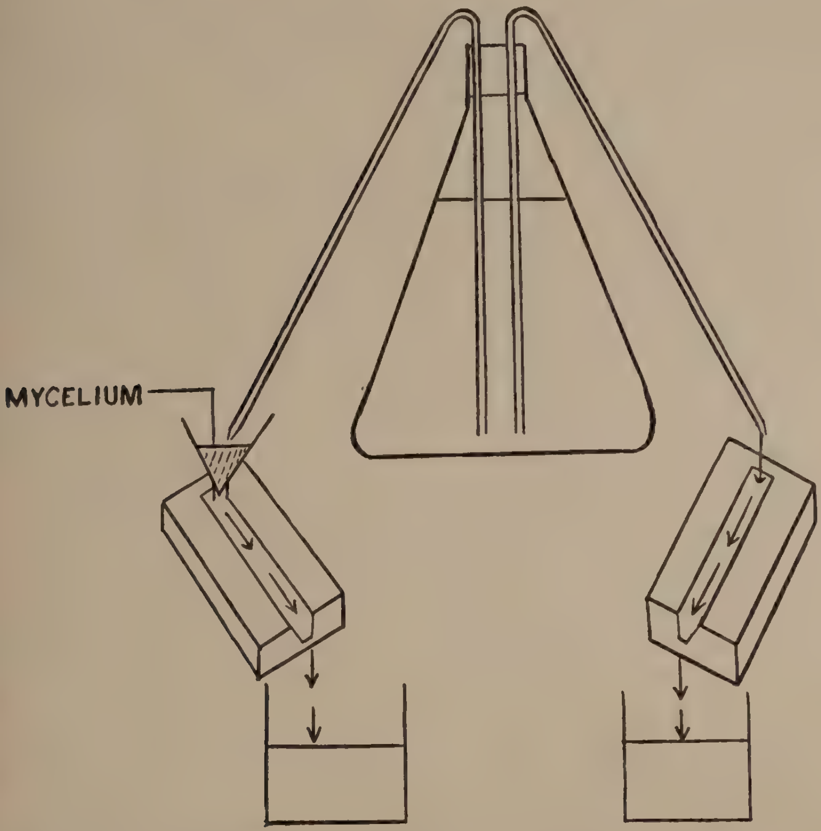

Fig. 8 The arrangement for the experiment in which used Rhizopus medium flowed over spores partially imbedded in the floors of agar channels. The experimental preparation is on the left, with the medium washing through the old mycelium just before passing over the spores. The control, on the right, gets medium which has not had recent contact with the mold.

DISCUSSION

There is reason to believe that the staling reaction is a generally occurring aspect of growth in molds and that the staling substance is intimately involved in the growth process. The staling tropism has been demonstrated in species of Mucor, Aspergillus, and Penicillium on the plastic plates used in this study. It has further been shown that the staling substance from any of these molds will elicit the negative tropic response in germinating spores of Rhizopus. It seems probable that a comprehensive survey would show that filamentous molds, generally, exhibit a staling reaction of greater or lesser intensity.

It would be desirable to learn something of the chemical nature of the staling substance, but this material cannot be approached by the conventional methods of purification and identification because of its extreme instability. Two other methods have been employed in this study, but without great success. Substances and mixtures suspected of being or containing the staling substance were applied to the opposite layers in tropism experiments to see whether they would alter the orientation of spores in the test region. The results were negative for all materials tried. Secondly, environmental conditions which would alter the form or activity of certain types of substances were varied to see whether these caused any change in the staling reaction. Experiments were done with various degrees of aeration and at various acidities. The results of these studies indicated that the staling substance is not highly volatile and that the reaction is qualitatively the same through the pH range in which the mold can grow (pH 3 to pH 7.3).[10]

Another possible method of getting at the chemical nature of the staling substance suggests itself. It is known of some molds that the same strain will show strikingly different growth patterns on different media. A mold which remains confined to a small area around the point of inoculation on one medium will be seen to spread throughout the dish on another. It seems possible that in some of these cases the difference in appearance is based on the success or failure of the staling tropism. If such a strain could be found, then an attempt could be made to correlate one component of the medium with the performance of the staling reaction. In this way, precursors of the staling substance might be discovered.

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[10] The synthetic medium used in these experiments had a pH of 4.3. In the later experiments it was buffered to pH 5.8 with Na_2HPO_4, as this gave better growth.

When Miyoshi (1894) studied the tropic responses of molds, he concluded that they turned toward a variety of nutrients and that they turned away from poisons and other caustic materials. He must have envisaged the growing tip as possessing something approaching a sense organ, guiding the hypha into supplies of food and away from anything harmful. More recent work has indicated that the chemotropic sensitivity of molds is very limited. In the course of this study, a large number of compounds and mixtures have been tested for tropic action on Rhizopus. Outside of the staling reaction, no true tropic response has been detected to any of the materials tested. These findings substantiate the belief that staling substance causes orientation by playing a direct role at the site of growth. It therefore seems that further study of the staling reaction may tell us not only something of tropic responses generally, but also something about the process of growth.

The author wishes to express his gratitude to Dr. John T. Bonner, under whose stimulating guidance this work has been done.

SUMMARY

1. Germinating spores of *Rhizopus nigricans* grow in an oriented manner away from other spores and hyphae into regions of more sparse population. This behavior is called the "staling reaction," and it is a negative tropic response to some product of the mold's metabolism.

2. The active stimulus for the staling reaction has not been demonstrated in used medium from which the mold has been removed. However, it has been demonstrated that the staling reaction is caused by a free-diffusing factor other than heat,

and it is therefore concluded that this stimulus, called the "staling substance," is an unstable chemical material.

3. Orientation is accomplished, not only by hyphal turning, but by oriented emergence of the germ tube as well.

4. The germinating spores produce growth factors which accelerate growth in crowded regions, but the evidence does not support the possibility of the staling substance itself being such a growth factor, eliciting orientation by differential growth acceleration. The hypothesis is offered that the staling substance acts by strengthening cell walls in its presence and thus preventing enlargement in such regions. The experiments thus far substantiate this view. It is suggested that the staling substance elicits tropic turning by direct intervention in the growth process.

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THE IMMOBILIZATION OF ADENOSINE TRIPHOSPHATE IN THE BIOLUMINESCENT REACTION[1]

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THREE FIGURES

When adenosine triphosphate (ATP) is added to a non-luminous aqueous extract of firefly lanterns, a bright flash of light is emitted which rapidly decays to a low steady state intensity (McElroy, '47; McElroy and Strehler, '49). The light intensity of this mixture can be increased by a second addition of ATP, suggesting that the latter is the limiting factor for light production. Considerable evidence has been presented, however, which suggests that most of the ATP is still present, but, by some unknown reaction, has been made unavailable for maximum light production (McElroy, '51). Since inorganic pyrophosphate or triphosphate greatly stimulates light production after the addition of ATP, it has been suggested that the former compounds are displacing the ATP from its "inactive" site, and thus making it available for light production. The present report is concerned with the purification of the two luminescent components and a study of their effect on the immobilization of ATP for light production.

EXPERIMENTAL

Purification of luciferase. Five grams of the dried lanterns of *Photinus pyralis* were ground with sand and extracted three times with a total volume of 100 ml H_2O. The pH of the extract was adjusted to 8 with NaOH and the solution placed in the deep freeze. When the solution had frozen completely, it was then thawed and centrifuged for 30 minutes at 0°C. The inactive precipitate was discarded. Twenty-five milliliters of a calcium phosphate gel (16.7 mg/ml) was centrifuged and the supernatant discarded. The extract was then thoroughly mixed with the gel and the pH was adjusted to 8. After 15 minutes the mixture was centrifuged and the gel discarded. The supernatant (prep. B) was considerably more active than the crude extract. Ninety milliliters of the calcium phosphate gel was centrifuged and subsequently mixed with 90 ml of preparation B. The pH was maintained at 8. After 15 minutes the mixture was centrifuged and the supernatant was discarded. In this latter step most of the luciferase was adsorbed onto the gel while the majority of the luciferin remained in the supernatant. To remove the residual luciferin as well as inactive protein the gel was washed twice with cold alkaline water and then by a 2% solution of $(NH_4)_2SO_4$· (pH 8). Elution of the enzyme was obtained by washing the gel twice with a 7% solution of $(NH_4)_2SO_4$ at pH 8 (prep. C). The final volume of combined eluates of preparation C was 95 ml. Preparation C was then fractionated with $(NH_4)_2SO_4$ in successive steps of 10% saturation up to 50% saturation and then in units of 2 to 3% saturation up to 65%. The pH during this procedure was at 8. The major part of the active enzyme was recovered between 57–65% $(NH_4)_2SO_4$ saturation. The latter precipitate was dissolved in 25 ml of water (prep. D) and the enzyme was readsorbed onto calcium phosphate gel as described above. The supernatant was discarded. The enzyme was eluted with 7% $(NH_4)_2SO_4$ at pH 8 (prep. E) and precipitated by adding solid $(NH_4)_2SO_4$ to 70% saturation (pH 8). The precipitate was dissolved in 5 ml of H_2O and the pH was adjusted to 8 (prep. F). A further treatment of preparation F with the low concentration of calcium phosphate gel removed some inert protein (prep. G). The activity of the various fractions is summarized in table 1. In this procedure the enzyme was purified approximately 70 times on a protein basis with a total recovery of 15%. In addition the preparation is completely free of luciferin and under these conditions no light is emitted upon the addition of ATP. The enzyme preparation shows a characteristic absorption peak at 279 mμ. The final preparation is also essentially free of pyrophosphatase, an enzyme which occurs in unusually high concentrations in the firefly lanterns (McElroy et al., '49). The enzyme is stored at — 17°C. where it can be maintained without any great loss of activity for several weeks. Repeated thawing and freezing increases the rate of inactivation.

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[1] Contribution no. 25 of the McCollum-Pratt Institute.

TABLE 1

Purification of firefly luciferase. Preparation of the crude extract and the details of the various steps are described in the text. Protein analyses were determined by the method of Herriott ('41) as modified by Lowry et al. ('51). The light intensity was measured by an electron multiplier tube (General Electric 931A) with approximately 90 v. on each stage.

PREPARATION	LIGHT UNITS PER ML-V.	PROTEIN MG PER ML	SPECIFIC ACTIVITY VOLTS PER MG PROTEIN
A. Crude	57	10.1	5.7
B. Supernatant 1st $Ca_3(PO_4)_2$ gel	166	6.3	26
C. Eluate 2nd $Ca_3(PO_4)_2$ gel	80	0.82	98
D. 57–65% $(NH_4)_2SO_4$ precipitate	105	0.60	175
E. Eluate 3rd $Ca_3(PO_4)_2$ gel	92	0.33	279
F. 70% $(NH_4)_2SO_4$ precipitate	210	0.627	335
G. Supernatant 4th $Ca_3(PO_4)_2$ gel	182	0.465	391

Purification of luciferin. Crude preparations of luciferin were obtained by dialyzing an aqueous extract of firefly lanterns against a large volume of water. The dialysate was then concentrated by vacuum distillation. The pH of the concentrated dialysate was adjusted to 3.5 and extracted twice with one-half the volume of ethyl acetate. All of the active luciferin passes into the ethyl acetate leaving a large number of other fluorescent compounds in the aqueous phase. The ethyl acetate was removed by vacuum distillation and the active luciferin was dissolved in a small volume of H_2O. The luciferin preparation was further purified by absorbing the latter on an acid (2 NHCl) treated Dowex 50 column (Mesh size less than 80). The column was washed thoroughly with 2 NHCl and finally water. The luciferin was slowly developed on the column by a weak solution of NH_4OH (1.5%). The luciferin migrated down the column in a sharp band and was finally eluted. The luciferin could be readily followed on the column by its brilliant yellow-green fluorescent band. The eluates containing the active luciferin were again extracted with ethyl acetate and the latter was concentrated by vacuum distillation. The luciferin was finally concentrated in water and used in this form for most of the enzyme studies. The physical and chemical characteristics of the purified luciferin have not been completed. Although the fluorescent characteristics do not appear to be significantly different from those previously reported (Strehler and McElroy, '49), the absorption spectrum is considerably different. The latter difference may be due primarily to the elimination of a second yellow-green fluorescent compound which results from the oxidative inactivation of luciferin. At neutral pH luciferin has two characteristic absorption maxima, one major peak at 330 mμ and a secondary peak at 263 mμ. The exciting wavelength for fluorescence corresponds to the adsorption peak at 330 mμ. The concentrated luciferin is slightly yellow in alkaline solution but changes to a colorless solution in weak acid. In the former case the fluorescence upon ultraviolet activation is an intense yellow-green, whereas in the latter case the fluorescence changes to a pale red. The luciferin can be maintained for several weeks without an appreciable loss of activity either frozen in the aqueous solution or in the dried state. In aqueous solution at pH 3.5 and 100°C. complete inactivation occurs in 15 minutes and approximately 50% loss of activity in 5 minutes. At pH 10 less than 5% inactivation occurs in 20 minutes at 100°C. The inactive luciferin can be removed from the active by extraction with ethyl acetate at pH 3.5. Under these conditions only the latter is removed from the aqueous phase.

Luciferin concentration and light intensity. The following reaction mixture was employed for the measurement of light response to varying concentrations of luciferin: 0.5 ml of 0.01M $MgSO_4$, 0.2 ml Enzyme (prep. F), 1 ml of 0.0015 M ATP (Schwarz), luciferin and sufficient 0.01 M Na_2HPO_4 (pH 7.5) buffer to give a final volume of 10 ml. In the presence of this excess of ATP and Mg^{++} ions, the intensity of light from the purified enzyme preparation depends upon the luciferin concentration. Figure 1 illustrates this relationship. The initial maximum light response is plotted against the luciferin concentration. In these preparations the light intensity rapidly decreases to a low residual level indicating, as previously shown, the removal of "active" ATP from the luminescent reaction. The rate of decrease of the light intensity, however, is inversely proportional to the luciferin concentration, i.e. at high luciferin concentrations the light intensity decreases faster than at low concentrations of luciferin. This variation in the rate of light intensity decay results in an inverse relationship of light intensity to the luciferin concentration after the reaction has proceeded for two to three minutes. However, upon the addition of inorganic triphosphate the immediate light response is proportional to the luciferin initially added. The rate of the light intensity decay can be obtained by taking the slope of the straight line which results from plotting the log, light intensity versus time for the first 10 seconds of the reaction. By plotting the rates obtained against the corresponding luciferin concentration the relationship shown in figure 1 is obtained. The results demonstrate clearly that the rate at which the light intensity decreases depends upon the concentration of luciferin in much the same way as the initial light intensity depends upon this factor. Similar results have been obtained for varying concentrations of Mg^{++} ions, i.e. the steady state level of luminescence was higher with a low Mg^{++} ion concentration.

Additional evidence has been obtained concerning the need for luciferin in making ATP unavailable for maximum light production. If ATP is added to a reaction mixture containing all components except luciferin, no light is emitted. After two minutes when sufficient luciferin is added to saturate the system, a bright flash of light occurs, similar to that normally obtained when luciferin is initially present (fig. 2). On the other hand, if a small amount of luciferin (sufficient to give 20% of maximum light intensity) is initially added along with ATP, the second addition of a large luciferin con-

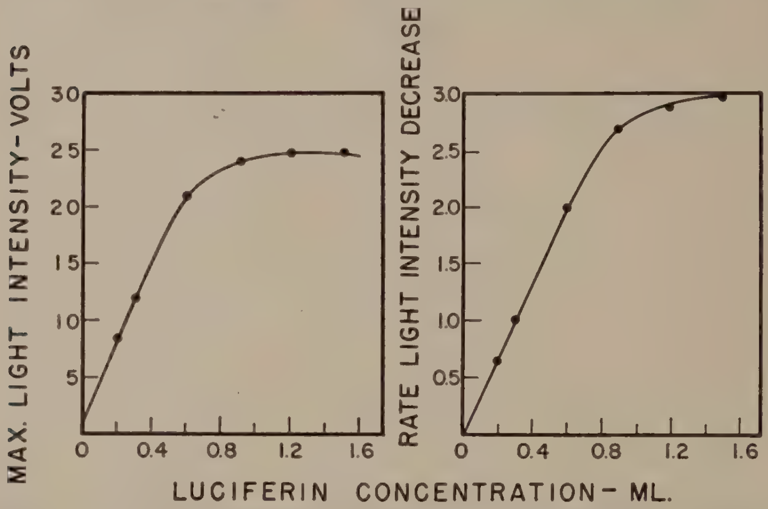

Fig. 1 The effect of luciferin concentration on the initial light intensity and the rate of light intensity decrease. The reaction mixture contained 0.5 ml of 0.01 M $MgSO_4$, 0.2 ml enzyme (prep. F), 1 ml of 0.0015 M ATP, varying amount of purified luciferin and sufficient phosphate buffer (0.01 M pH 7.5) to give a final volume of 10 ml. The curve at the left describes the initial light intensity at varying luciferin concentrations whereas the curve at the right describes the rate of light intensity decrease as a function of luciferin concentration.

centration does not raise the light intensity to its maximum. Upon the addition of inorganism triphosphate, however, the normal response is obtained, indicating that the ability to mobilize the ATP is unimpaired. These results in addition to the ones cited above clearly establish the fact that luciferin is essential for the rapid immobilization of ATP for light production.

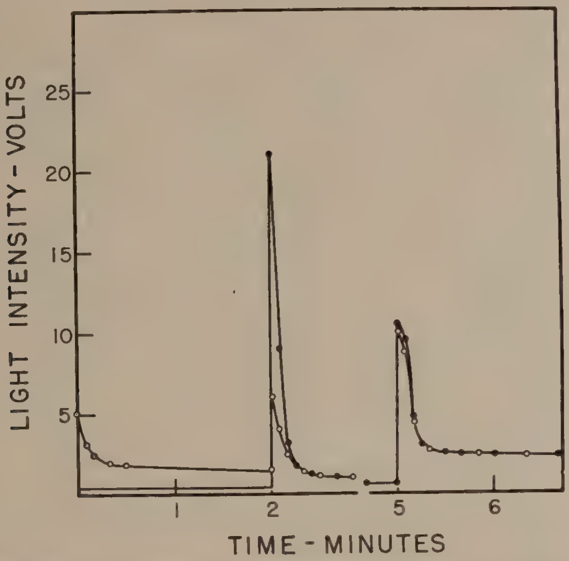

Fig. 2 The effect of luciferin on the immobilization of ATP in the luminescent reaction. Solid circles — the reaction mixture was the same as that described for figure 1 except that no luciferin was added initially. At two minutes 1 ml of luciferin solution was added and the light response recorded. Open circle — same reaction mixture as above except 0.1 ml of luciferin was added at zero time. At two minutes an additional 0.9 ml of luciferin was added. At 5 minutes 0.5 ml of 0.001 M sodium triphosphate was added to both reaction mixtures.

Effect of Ca^{++} ions. It has been reported previously that calcium inhibits the utilization of ATP for light production in the firefly extracts (McElroy and Strehler, '49). It was of interest therefore to determine the effect of this ion on the immobilization of ATP. The results obtained in a series of experiments indicated that calcium interfered with not only the initial light intensity but also its rate of decay. The higher the calcium ion concentration the slower was the rate of light intensity decrease. Since previous results indicated that calcium competed with magnesium and since the latter is apparently necessary for the immobilization of ATP such a result is to be expected. With a decreased rate of light intensity decay a greater total light output is obtained with increasing concentrations of the calcium ion. The results are presented in figure 3. It is evident that with the calcium ion concentration employed in these experiments the decay of the light intensity is affected much more than is the initial flask. The total light is represented by the area under the curve obtained from a plot of light intensity versus time in which the observations were continued until the light intensity reached the steady state level. The intensity for the latter was ap-

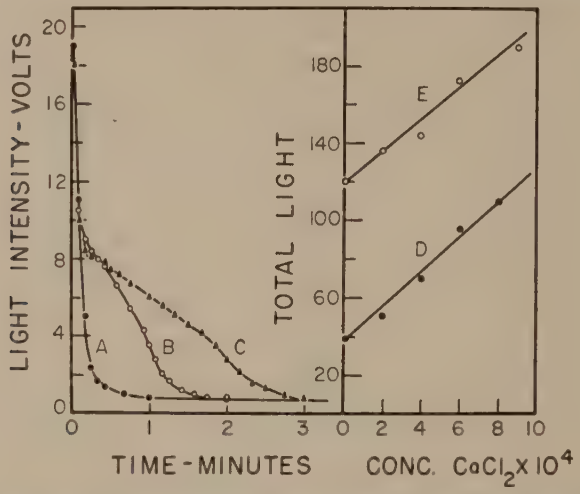

Fig. 3 The effect of calcium on the light intensity and the total light emitted by the purified luminescent system. Same reaction mixture as described in figure 1. Curve A — control — no calcium; curves B and C contain a final concentration of $CaCl_2$ of 4 and 6×10^{-4} molar respectively. In the figure at the right the total light refers to the area under the curves taken up to the time the light intensity reaches the steady state level. Curve E relates total light to ATP added initially, whereas curve D refers to the response to inorganic triphosphate (final concentration — 5×10^{-4} M).

proximately the same for all reactions irrespective of the calcium ion concentrations. It is also significant that the response to inorganic triphosphate is similar to the initial light response obtained with ATP.

DISCUSSION

The evidence which has been presented in this and previous publications demonstrates that when adenosine triphosphate is added to the luminescent system it is rapidly immobilized and is available to only a limited extent for light production. By partially purifying and separating the luciferase and luciferin from each other it has been possible to demonstrate the importance of the latter in the immobilization process. Apparently the ATP is rapidly removed long before the luciferin is utilized for light production, since a normal light response is obtained if either additional ATP or inorganic triphosphate is added to the reaction mixture. Since both magnesium and luciferin are essential for the immobilization of ATP as well as light production it seems likely that the two processes are closely linked, a suggestion which is in keeping with all of the kinetic data. The light-emitting system would presumably be an intermediate in the immobilization reaction in which oxidation of the luciferin would occur. If oxidation failed to occur, as under anaerobic conditions, the ATP is then converted into an inactive form. The experimental results suggest that the inactive ATP complex is unstable and is continually decomposing to give rise to the normal intermediates necessary for light production and that compounds such as inorganic triphosphate accelerate the mobilization of ATP presumably by a displacement reaction. It is possible that ATP is converted into a form which acts as a competitive inhibitor, while inorganic triphosphate as well as free ATP act as effective antagonists.

The results with calcium ions clearly indicate that as long as the immobilization of ATP is prevented, a greater light output can be obtained for a given concentration of ATP.

This and other results support the idea presented above that the light emitting particle is an intermediate in the reaction leading to the immobilization of ATP. Experiments designed to directly demonstrate the formation of an altered form of ATP have not been successful.

SUMMARY

1. The separation and partial purification of firefly luciferase and luciferin is described.

2. Using purified components it is possible to demonstrate that the intensity of light emission in the presence of adenosine triphosphate, Mg^{++} and luciferase depends upon the luciferin concentration up to saturation.

3. The rate of light intensity decrease after adding ATP depends upon the luciferin concentration, indicating that the latter is essential in the immobilization of ATP for maximum light emission.

4. Calcium inhibits the rate of light intensity decay resulting in a greater total light emitted. The response to a delayed addition of inorganic triphosphate to the reaction mixture is also affected by the calcium ion concentration.

5. The results are discussed in relation to the hypothesis that the light-emitting particle is an intermediate in the reaction leading to the immobilization of ATP.

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HEATING OF ANIMALS BY ABSORBED SOUND ENERGY

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FIVE FIGURES

INTRODUCTION

There have been several reports describing the heating of animals by high energy level air-borne sound. Allen et al. ('48), Frings et al. ('48) and Frings and Senkovits ('50) have killed mice by exposing them to sound from a laboratory siren capable of producing 160–165 db ref 10^{-16} watts/cm^2 from 18 kcps to 25 kcps. The killing of white rats and guinea pigs in sound fields of frequencies from 1 to 18 kcps. is reported by Parrack and Eldredge ('48), Parrack et al. ('48) and Eldredge and Parrack ('49). Under special circumstances or with higher sound levels heat effects on the skin of man have been observed and are mentioned by most investigators (Allen et al., '48; Frings et al., '48; Parrack and Eldredge, '48; Parrack et al., '48; Eldredge and Parrack, '49).

In the present paper a theoretical explanation for all these phenomena is given.[1] It is shown that overheating of the animals because of the mechanical energy absorbed by the body surface in the intense sound field may be expected at the observed sound levels. In order to find the energy absorbed by the exposed animal the acoustic absorption coefficient of the body surface of white rats has been measured in the frequency range up to 6 kcps. (Section I.) By the use of these data and data for the heat balance of the animal dangerous sound levels and the time required for overheating have been calculated. (Section II.) These results are compared with previously reported data and also with new more accurate experimental observations (Section III) on rats. The good agreement between theoretical and experimental results confirms the interpretation that overheating caused by the absorbed sound energy is responsible for the death of the animals in each of the reported experiments.

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[1] The authors want to express their appreciation to Dr. Rudolf Thauer of the Aero Medical Unit, Naval Air Experimental Station, Philadelphia, Pennsylvania, for valuable discussions on the subject of this paper.

These results may serve as a guide for further experiments as well as indicate, for other species of animals, the order of magnitude of the sound level at which overheating will occur.

I. Measurements of the sound absorption coefficient for the white rat

The sound absorption coefficients for the body surface of white rats were measured with an impedance tube. Discussions of the validity of the measured absorption coefficient, of the theories of the two measuring methods and of the apparatus used for the measurements are given in previous reports by Gierke ('49) and Gierke ('50). Both the tube resonance method and the tube decay time method previously described were used and the results obtained with both methods were found to agree satisfactorily.

Two different circular areas ($S = 4.9\ \mathrm{cm}^2$ and $17.6\ \mathrm{cm}^2$) on the sides of anesthetized rats were used for the measurements. To maintain an airtight closure between the impedance tube and the test area of the animal, the fur surrounding the area to be measured was removed and petroleum jelly applied to the shaved skin. Except for the measurement of absorption coefficients for shaved animals, the entire closing surface of the animal that lay within the tube had a normal amount of fur.

The absorption coefficients were found to vary widely from animal to animal, sometimes by as much as 100%. This may be explained by differences in the fur thickness and size of the animals used. For a given animal it was found that the absorption coefficient for ruffled fur was as much as 30% more than for fur which was smoothed down. The hair length of the animals used for the measurements was about 8 mm.

The general dependence of the absorption on frequency is shown in figure 1, measured for an area $S = 4.9\ \text{cm}^2$. Curve 1 a shows the absorption coefficient, a, for normal intact fur on a rat's flank. It decreases from about 5% at 400 cps to a minimum of approximately 3% at about 1,500 cps and then increases with frequency throughout the frequency range measured up to about 12% at 6 kcps. In order to explain this curve the absorption coefficients for a shaved rat and for furred skin removed from the animal and supported on a rigid wall were measured. Typical measurements of these absorption coefficients are shown in curves 1 b and 1 c. For the shaved rat (curve 1 b) a varies inversely with frequency throughout the range measured. For the furred skin (curve 1 c), a increases with frequency. By comparing the three curves in figure 1 it seems quite clear that the absorption coefficient, a for the ani-

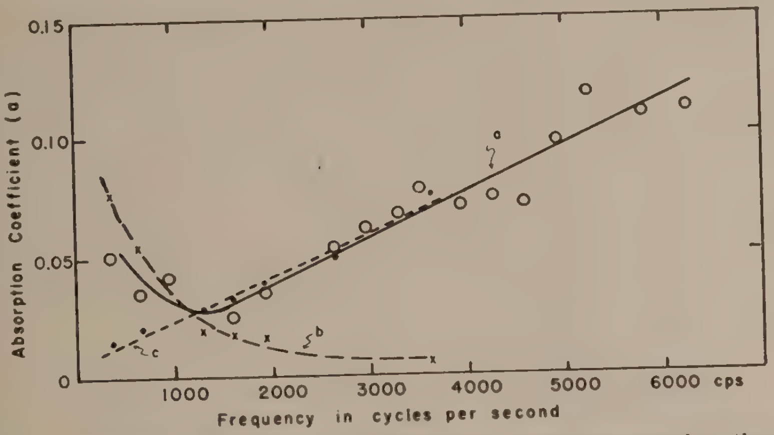

Fig. 1 Absorption coefficient, a, measured over an area $S = 4.9\ \text{cm}^2$ on the side of white rats as a function of frequency, f.

(a) Absorption of a rat with fur.
(b) Absorption of a shaved rat.
(c) Absorption of the furred skin supported by a rigid wall.
(Measurements were made on three different rats.)

mal with fur as shown in curve 1 a is in general a composite of curves 1 b and 1 c. An exact numerical agreement cannot be expected because the three curves are derived from different animals and show only the general relation. But it is obvious that for the animal with fur in curve 1 a the absorption coefficient below 1,500 cps depends largely on the physical properties of the underlying tissues and the rat's geometrical shape. These same factors determine the decrease of a with increasing frequency in curve 1 b. The result shown in curve 1 b is to be expected for the shaved rat's surface because this surface is, in general, similar to the human body surface overlying soft tissues, for which a also has been shown by Gierke ('50) to vary inversely with frequency. Above 1,500 cps a in curve 1 a is obviously determined by the same factor which determines the slope of the curve 1 c; that is, by the physical properties of the fur. A physical explanation for the shape of curve 1 c may be obtained by assuming a honeycomb structure, as an approximation for the fur, in calculating its theoretical absorption coefficient (Crandall, '26).

Whereas it may be assumed from the nature of the measured absorption coefficient of the fur, curve 1 c, that the sound energy which is absorbed in the fur is transformed there into heat, this assumption is not necessarily justified for the sound energy absorbed by an area of a shaved rat or by an area of a furred rat at low frequencies. Here the sound energy is transmitted through the animal in all directions and causes vibrations of other parts of the body surface from which energy may be radiated into the surrounding air or other objects in contact with the animal. However, under most circumstances and when the frequencies are not very low this amount will be very small. Therefore, it may be assumed that even at low frequencies (100–1,500 cps) most of the absorbed acoustic energy is damped in the tissue and appears as heat. But in the whole range, where we found a largely dependent on the underlying tissue we have to keep in mind, that the measured absorption coefficient will depend very much on the area over which it is measured and may be used only with caution. This question has been discussed in more detail by Gierke ('50) for the human body surface. For the rat, it is still more difficult because the area used in the measurements is not at all small compared to the body dimensions and any pressure applied to an area on the surface results in a deformation of the whole rat. So there is, for an instance, nearly no connection at all between the absorption coefficient measured with a tube on one side of the body and the absorption coefficient which we would get for the whole rat in a very low frequency sound field, where the rat

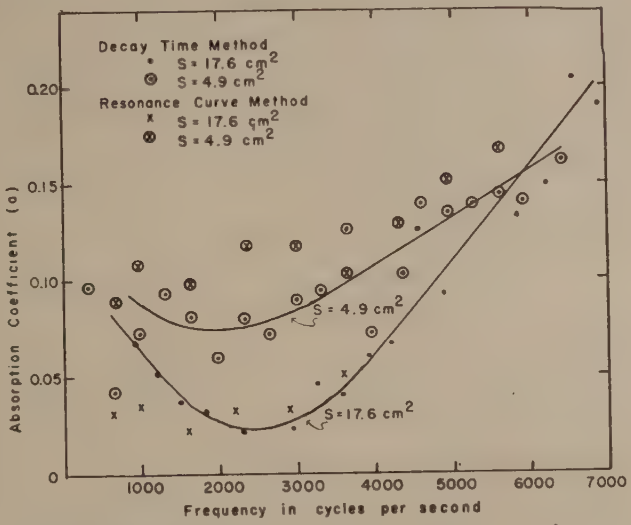

Fig. 2 Absorption coefficient, *a*, of a rat as a function of frequency, f. Test area S = 4.9 cm² and S = 17.6 cm².

would be compressed in the same phase from all sides. But we can be sure that the absorption coefficient per unit area for the whole animal will be always smaller than the absorption coefficient measured with our methods for smaller areas. The absorption coefficient measured on a small area at low frequencies, where the tissue and the body shape predominantly determine the absorption, thus gives us for the whole body an upper limit of absorption. At higher frequencies where *a* depends primarily on surface absorption in the fur, *a* should be independent of the area of measurement.

These assumptions are partly confirmed by the measurements shown in figure 2. Here a was measured as a function of frequency for areas of 4.9 cm^2 and 17.6 cm^2 by both the tube resonance method and the decay time method. The larger area shows a smaller absorption coefficient and a minimum at a higher frequency. But at still higher frequencies the two curves approach each other; this means, the absorption coefficient becomes less dependent on the measured area. These measurements were made on one animal which had one flank prepared for a 4.9 cm^2 area and the other flank for a 17.6 cm^2 area.

Comparison of figure 1 with figure 2 shows the remarkable difference in the data obtained from one animal to the other as well as the deviations of the single points measured. But for the approximate calculations of the present paper the absorption coefficients as measured are felt to be sufficiently accurate.

II. Calculation of the quantity of sound energy absorbed by the white rat and its heat balance in intense sound fields

Using the measured values of the absorption coefficient, the total energy absorbed by a rat in a sound field of given frequency and intensity may be calculated. Such a calculation must be an approximation because: (1) In most of the practical cases we do not know, exactly, the sound field in which we place the animal. There may be only one plane wave striking the animal from one side or we may have a more or less statistical distribution of sound waves traveling in all directions. (2) We do not know the diffraction pattern of the sound field around the rat, since this depends on the rat's shape and the specific impedance of its body surface.

We may consider the rat for this calculation to be an absorbing sphere exposed to plane sound waves of the intensity, I. Even when this simplifying assumption is made the total energy, E, absorbed by the rat is difficult to calculate. We must also know the specific impedance, $z = w + j q$, of the body surface in order to calculate the effective absorbing cross section S_e, of the assumed sphere which is required to express the total energy absorbed by the spherical surface by the equation $E = S_e\, a\, I$. The value of S_e varies remarkably with a change of the impedance, z, even when a is held constant. Especially when w is small compared to the characteristic impedance of air and q is a capacitative reactance, S_e varies considerably with frequency and resonance phenomena appear at certain frequencies. But practically we have observed by the measurements that the resistance w of the fur is never small compared to the characteristic impedance of air. Furthermore the resonances would probably be damped by the rat because the impedance of its surface is not as uniform as is assumed for the sphere. We shall not make exact calculations for S_e, which can be calculated, however, from the data of Lax and Feshbach ('48), and in order to simplify our problem we shall assume that S_e is constant for the whole frequency range and has the average value of half the surface of the sphere, S_s hence $S_e = \frac{S_s}{2} = 2\pi r^2$ (r = radius of the sphere). Tests by more exact calculations showed this value to be a good approximation for the impedance and absorption coefficients involved. On the other hand a change of S_e does not influence greatly the final results shown in figure 3. For instance changing the value of S_e by a factor of 2 shifts the curves of figure 3 only by 6 db. Therefore, we assume that the total energy absorbed by a rat from a plane sound wave, is given by

$$E = S_e\, a\, I \cong 0.5\, S_R\, a\, I \qquad (1)$$

where $S_R \cong S_s$ means the total surface of the rat. Another reason why our calculations can give only approximate results is the dependence of a on area as shown in figure 2.

For the next step in our calculation we conclude by comparison of curve (a) and (c) in figure 1 that at frequencies higher than 1.5 kcps nearly all the energy, E is absorbed by the fur and transformed there into heat. When the temperature of the fur rises the normal transfer of metabolic heat from the body to the surrounding air is diminished and the rat's body temperature starts to increase. Let us now calculate this rise in body temperature as a function of time by considering the equation for the heat balance of the rat's body. To simplify the problem we neglect all heat losses through evaporation and respiration. For the rat these losses are probably always a small part of the normal metabolic heat (Herrington, '40) and in this case are surely small compared to the heat dissipated by convection and radiation. Then the heat balance is given by the equation:

$$M + E = \frac{C_B \Delta T_B}{t} + \frac{C_F \Delta T_F}{t} + S_R \alpha_A (\theta_{AF_0} + \Delta T_F) \qquad (2)$$

$C_B = c_B G_B =$ Heat capacity of the body.
$c_B =$ Specific heat of the body.
$G_B =$ Weight of the body.
$C_F =$ Heat capacity of the fur.
$\Delta T_B =$ The increase in body temperature during exposure time t.
$\theta_{AF_0} = T_F - T_A =$ Initial difference between the temperature of the fur and the surrounding air at $t = 0$.
$\theta_{FB_0} =$ Initial difference between the temperature of the fur and of the body at $t = 0$.
$\theta_{FB} = \Delta T_F = \Delta T_B - \theta_{FB_0} =$ Difference between temperature of the fur and of the body at time t.
$\Delta T_F =$ The increase in fur temperature during exposure time t.
$\alpha_A =$ Coefficient of heat transfer (radiation and convection) from the surface of the fur to air.
$\alpha_B =$ Coefficient of heat transfer from the surface to inside of the body (rectal).
$M =$ Metabolic heat of the rat.
$S_R =$ Surface of the rat $\cong$ skin surface of the rat.
$t =$ Exposure time.

At the skin surface another equation applies:

$$S_R \alpha_B \theta_{FB} + M = \frac{C_B \Delta T_B}{t} \qquad (3)$$

which is the same as:

$$S_R \alpha_B \Delta T_F + M = \Delta T_B \left(\frac{C_B}{t} + S_R \alpha_B\right) + S_R \alpha_B \theta_{FB_0}$$

If we assume that M remains at approximately its initial value at $t = 0$ then M may be expressed as:

$$M \cong S_R \alpha_B \theta_{FB_0}$$

and

$$\Delta T_F \cong \frac{\Delta T_B}{S_R\,\alpha_B}\left(\frac{C_B}{t} + S_R\,\alpha_B\right)$$

With this expression we obtain from equation (1) by neglecting the obviously small heat capacity C_F of the fur:

$$\Delta T_B\left[\frac{C_B}{t} + \frac{\alpha_A}{\alpha_B}\left(\frac{C_B}{t} + S_R\,\alpha_B\right)\right] + S_R\,\alpha_A\,\Theta_{AF_0} \cong M + E$$

From this we obtain the time t, necessary to raise the temperature of the rat's body by ΔT_B.

$$t \cong \frac{C_B\,\Delta T_B\left(1 + \frac{\alpha_A}{\alpha_B}\right)}{M + E - S_R\,\alpha_A\left(\Theta_{AF_0} + \Delta T_B\right)} \qquad (4)$$

The quantities of this equation are known approximately. Even if α_A, α_B and M are somewhat changed by a change in temperature, the effect on t in equation (4) is not very large. Let us assume for an average rat after Brody ('45).

$G_B = 300$ grams.
$S_R = 0.041\ m^2$ (the normal value is here enlarged by a thickness of the fur of 0.5 cm). This corresponds to a sphere with $r = 5.75$ cm.
$c_B = 0.83$ Cal./kg/°C.
$\frac{M}{S_R} = 31.9$ Cal./hr./m².

The values for α are taken from Herrington ('40). Calculating with his value of 225 Cal./day/m²/°C. for the heat conductivity of the fur we obtain:

$\alpha_A = 150.7$ Cal./m²/day/°C. $= 6.28$ Cal./m²/hr./°C.
$\alpha_B = \alpha_{\text{(rectal-skin)}} = 272$ Cal./m²/day/°C. $= 11.35$ Cal./m²/hr./°C.

The values for α_A and α_B were measured at an environmental temperature of 26.7°C. They must be used as the basis for our calculations because it is not known how α_B may be changed by the kind of heat load encountered in the sound field. Higher values for α_B were observed at higher environmental temperatures. Should a higher value for α_B also apply in the present circumstances, the overheating process in the rat would be accelerated accordingly.

If we assume a room air temperature of about 27° then Θ_{AF_0} will be about 10°C. If we want to get as the final result from equation (4) the time t_L required for lethal overheating of the animal in the sound field, we must insert for ΔT_B a rise in the body temperature assumed to be fatal to the animal. This may be a rise in body temperature from 37°C. to 42°C. so that ΔT_B is 5°C.

Using all these values in equation (4) the time required for lethal overheating in the sound field may be calculated for any amount of energy, E, absorbed by the rat. By the use of equation (1) this lethal time can be given directly as a function of the sound intensity, *I*, in the plane wave striking the rat, as it is plotted in figure 3. The absorption coefficient, *a* on which t_L depends, is the parameter.

The curves show that, when *a* is held constant, exposure to plane sound waves of any level up to a certain level, which we shall call the minimum lethal level, will not produce the lethal temperature rise of 5°C. even when the exposure time is infinite. At the minimum lethal sound level the time required for the body temperature to rise by 5°C. is just infinite; i.e. $t_L = \infty$. Above the minimum lethal sound level the lethal time, t_L, decreases rapidly with increasing sound intensity. At intensities 10 db above the minimum lethal sound level the lethal time is shorter than 10 minutes. The lethal sound levels for the rat lie above 150 db for the range of sound absorption coefficients measured on the rat (less than 0.20).

In experiments with rats in intense sound fields our assumptions are difficult to realize exactly: the sound field is not a plane wave, the diffraction pattern is more complicated than that assumed, the animal has an irregular shape and, if not anesthetized, moves during exposure. In addition, individual variations in age, weight, size, fur thickness and normal body temperature will prevent exact comparison from animal to animal. From the steep slope of the curves in figure 3 it may be expected that experimentally determined lethal times could vary within quite wide limits. However, in general the curves of figure 3 must give rather accurate lethal limits, for they are

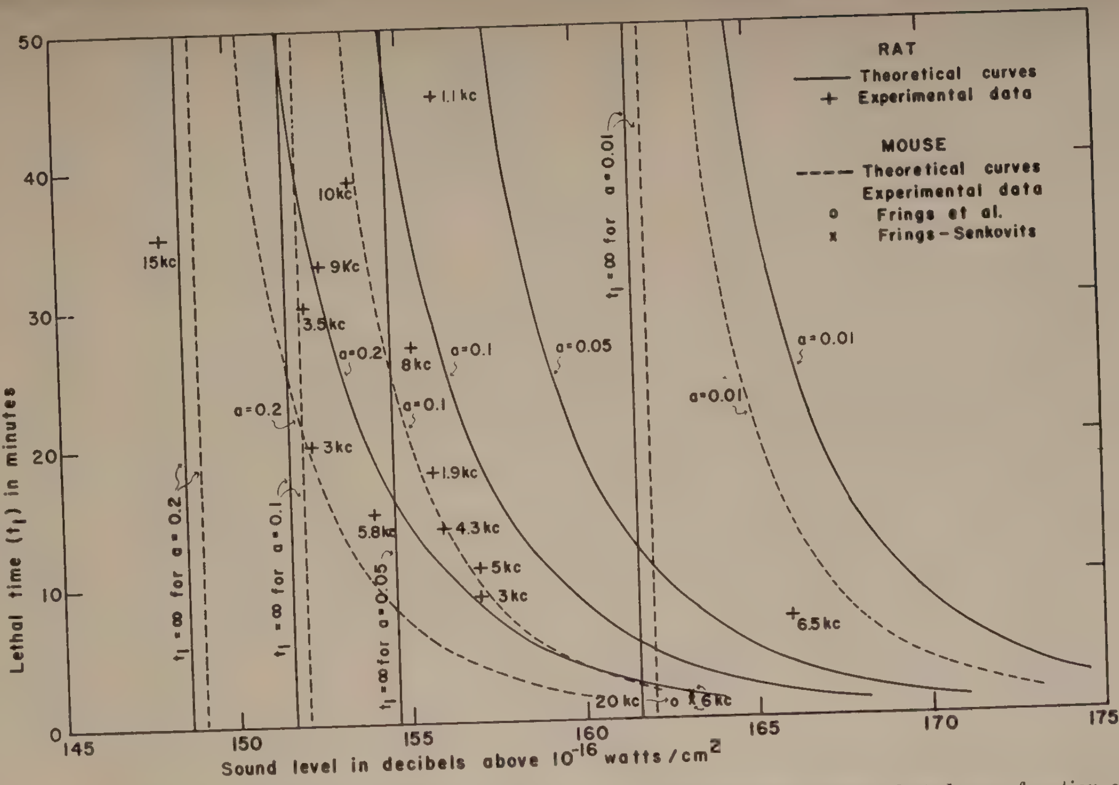

Fig. 3 Calculated curves for the time of exposure t_L, required to kill the rat or the mouse, plotted as a function of the intensity, I, of a plane sound wave.

changed only slightly if we make any reasonable changes in our assumed constants. For example, twice the value for α_A would shift the curves by only 4 decibels. Or if we take into account heat loss by evaporation (measured by Herrington ('40) as about 15% of the normal metabolic heat) and assume that the rat may dissipate 100% of its normal metabolic heat by sweating we get a shift in the curves of figure 3 of only two decibels toward higher sound levels.

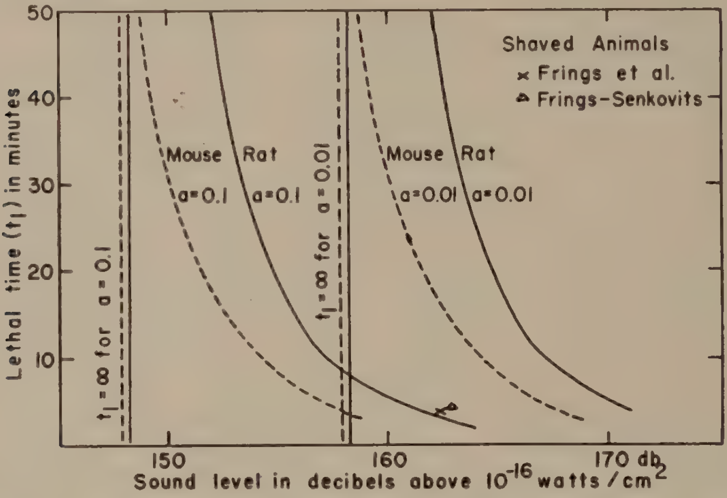

Fig. 4 t_L as a function of *I* for the shaved rat and shaved mouse. Compare with figure 3, furred animals.

To apply to the shaved rat formula (4) is changed to:

$$t_L \cong \frac{C_B \, \Delta T_B}{M + E - S_R \, \alpha_{AB} \, (\Theta_{AB_0} + \Delta T_B)} \qquad (5)$$

α_{AB}, here, is the total rectal-air heat transfer coefficient. It is calculated using the data from Herrington ('40) for the rat without fur.

$\alpha_{AB} = 96$ Cal./m²/day/°C. $= 4$ Cal./m²/hr./°C.
$\Theta_{AB_0} =$ Initial difference between the temperature of the surrounding air and of the body at $t = 0$.

Lethal time, t_L, as a function of sound level as described by formula (5) is plotted in figure 4. If the shaved rat had the same absorption coefficient as the furred rat, i.e., about, 0.1, the lethal sound intensity level would be 4 db below that for the furred animal. However, for frequencies above 1,000 cps the shaved rat shows a much smaller value for a than the furred rat (fig. 1). Therefore the lethal sound level for a shaved rat will be much higher than that for the furred animal. At frequencies below 1,000 cps the lethal sound level for the furred and shaved rats will be approximately the same.

Again using Herrington's data it is possible to calculate from formula (4) curves describing lethal times and sound levels for mice and guinea pigs. The dotted lines in figures 3 and 4 show the results of such calculations for the mouse. Using the data from Herrington ('40) and Brody ('45) that:

$$G = 0.025 \text{ kg.} \qquad a_B = 8.85 \text{ Cal./m}^2\text{/hr./°C.}$$

$$\frac{M}{S} = 34 \text{ Cal./m}^2\text{/hr.} \qquad a_{AB} = 4.0 \text{ Cal./m}^2\text{/hr./°C.}$$

$$a_A = 6.75 \text{ Cal./m}^2\text{/hr./°C.} \qquad S = 0.008 \text{ m}^2.$$

it is obvious that in spite of the different sizes of the animals the minimum lethal sound level remains about the same. The difference between the two cases is that when this minimum lethal level is exceeded the smaller animals will be overheated faster.

III. Experimental data on white rats compared with calculated limits for overheating

In earlier experiments white rats were exposed to the sound field of a laboratory siren at intensities sufficient to cause death by overheating (Parrack and Eldredge, '48). The first experiments were performed with unanesthetized animals in a small cage that served to confine the animal to the central, more intense, portion of the sound field. Fur and rectal temperatures were measured with copper-constantin thermocouples as accurately as possible on active animals. The variations of these sound fields were kept within ± 4 db by measurement with calibrated crystal microphones. Frequency was controlled by controlling of the rpm of the siren.

The results of this series of experiments, previously reported (Parrack and Eldredge, '48), are summarized in tables 1 and 2. The data in table 2 are presented separately because for these animals it was found possible to hold the siren output constant within ± 1.5 db.

TABLE 1

FREQUENCIES	SOUND LEVEL	FUR TEMP.	RECTAL TEMP.	TIME TO DEATH
1,100 cps	156 ± 4 db	42°C.	41°C.	45 min.
3,000 cps	157 ± 4 db	..	42°C.	9 min.
5,000 cps	157 ± 4 db	43°C.	63°C.	11 min.

A first analysis of these data and of similar data for the guinea pig indicated that frequencies around 3,000 cps were especially effective for overheating these animals. Subsequent examination of the siren sound fields with a panoramic sound frequency analyzer (Eldredge, '48) revealed that at certain frequencies, notably 3,000 cps, the siren cavities became resonant and produced a sound field in which the second harmonic was as great or greater than the fundamental. Thus it is not known for the data in tables 1 and 2 that the indicated frequency was at all times the principal frequency component of the sound field.

TABLE 2

FREQUENCIES	SOUND LEVEL	FUR TEMP.	RECTAL TEMP.	TIME TO DEATH
2,000 cps	152 ± 1.5 db	39°C.	39°C.	60 min. (living)
3,000 cps	152 ± 1.5 db	39°C.	41°C.	20 min.
3,500 cps	152 ± 1.5 db	39°C.	42.5°C.	30 min.
4,000 cps	152 ± 1.5 db	42.5°C.	41°C.	60 min. (living)
5,000 cps	152 ± 1.5 db	33°C.	39°C.	60 min. (living)

A further weakness in the above data is the inaccuracy with which temperature readings could be made on active animals. The results did show, however, that a small fur-bearing animal such as the white rat could be killed by sound fields of these frequencies and intensities in these time intervals and with elevations of body temperature sufficient to account for the deaths. It was felt that, if similar results were obtained using anesthetized rats, the effect of the anesthesia on temperature regulation could be neglected while the physical measurements of the experimental conditions were made more precise.

In a new series of experiments rats anesthetized with pentobarbitol sodium and occasional supplementary ether were suspended on a coarse mesh wire screen in a fixed position in the sound field. The frequency and intensity of the sound field were measured, at the animal position, with a calibrated crystal microphone and a panoramic sound frequency analyzer both before placing the animal in the field and after removing it. The same equipment was used to monitor the sound field at a point 1 inch above the rat throughout the exposure. In this way it was possible to know that the harmonics of the exposure frequency were at least 6 db below the fundamental and to control the output of the siren to within ± 1.5 db.

With the anesthetized rats it was relatively easy to affix copper-constantin thermocouples in the rectum and at the base of the fur and to measure these temperatures as frequently as desired. A summary of the exposure frequencies and sound levels, rectal and fur temperatures and the exposure times is given in table 3.

The data in table 3 are not sufficient to prove all the implications of the calculations based on the measured absorption coefficients. The exposure sound levels were purposely chosen to be just above or just below the lethal level. It seems clear that even small differences in sound level, may be critical here. The data also show a slight trend toward increasing energy absorption by the fur as the frequency increases.

To compare these experimental data with the calculated data of section II, as shown in figure 3, we must assume that the measured sound pressures are those of a plane sound wave. This assumption is justified by the fact that the sound field was generated in an anechoic room where reflection of the sound and the formation of standing wave patterns were minimal.

On the other hand, the output of the siren is focused on a central axis and a true plane wave sound field does not exist. However, the sound fields more nearly meet the requirements for plane wave fields, than for fields of random sound wave direction. The crosses in figure 3 show the relationship of the experimental results in tables 1–3 to the theoretical curves for various absorption coefficients. The measured lethal times and intensities place these points in a range of absorption coefficients of 10 to 20%. The measured values of the absorption coefficient, *a*, in figure 2 were found for areas of only 4.9 cm^2 and 17.2 cm^2 and not for the whole animals. However, considering all the possible inaccuracies in the theoretical calculations and the physiological assumptions, particularly in the value for body temperature increment, ΔT_B (anesthetized animals frequently started with lower rectal temperatures) the experimental results fit the theoretical curves very well. In any event the correlation is good enough to confirm the belief that heat produced by sound energy in these animals is sufficient to account for their deaths.

TABLE 3

FREQUENCIES	SOUND LEVEL	FUR TEMP.	RECTAL TEMP.	TIME TO DEATH
				min.
1,100 cps	156.5 ± 1.5 db	37.5°C.	37.5°C.	60 (alive)
1,900 cps	155.7 ± 1.5 db	46	42.5	18
4,300 cps	156 ± 1.5 db	45.5	41	14
5,800 cps	154 ± 1.5 db	55	42	15
6,500 cps	166 ± 3. db	..	..	7
6,900 cps	152.4 ± 1.5 db	37.5	38	45 (alive)
8,000 cps	152.8 ± 1.5 db	40	41	60 (alive)
8,000 cps	155.2 ± 1.5 db	47	42.5	27
9,000 cps	152.6 ± 1.5 db	44	41.5	33
10,000 cps	151.8 ± 1.5 db	41.5	41.5	70 (alive, in shock died in cage over night)
10,000 cps	153.5 ± 1.5 db	44.5	41	39
15,000 cps	148 ± 1.5 db	43	41	35
18,500 cps	143.4 ± 1.5 db	34	34	45 (alive)

Further confirmation that the heat is the decisive if not sole factor may be obtained by comparing the fur and rectal temperatures as a function of time for those animals which survived and for those animals which died. These values are plotted in figure 5 for an animal which died during exposure to 10,000 cps at 153.5 ± 1.5 db and for an animal which survived an exposure to 6,900 cps at 152 ± 1.5 db for 45 minutes. It can be seen that the animal which survived had reached a stable heat equilibrium with his environment at a temperature compatible with life. In the animal that died, the absorption of sound energy with conversion of that energy to heat in the fur was so great that a stable heat balance was impossible at a temperature compatible with life. Observation of surviving animals for periods as long as a month revealed no abnormalities except for deafness. Since the animals which were exposed to sound levels just below the critical heat producing level appeared to be physically normal following exposure, it does not appear likely that any other characteristic of the sound field was responsible for the lethal effect on the animals which died.

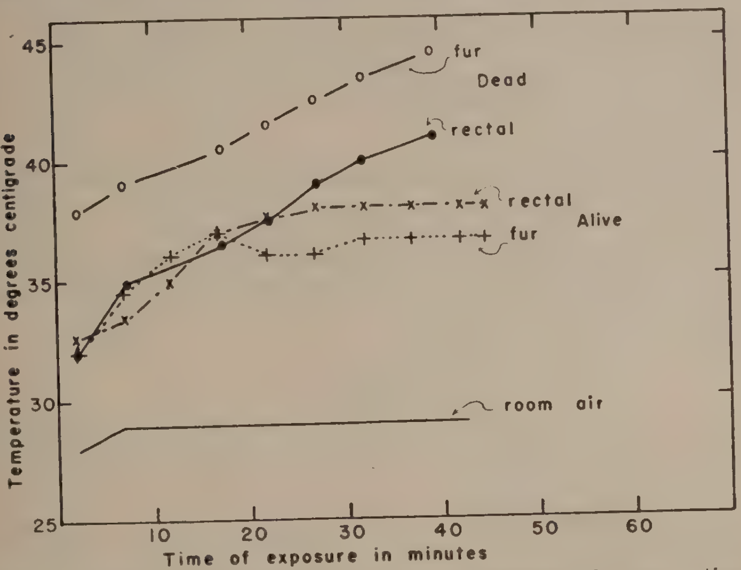

Fig. 5 Rectal and fur temperatures, rat, as a function of exposure time.

The white mice exposed by Allen et al. ('48) and Frings et al. ('48) to a sound level of 160–165 db at approximately 20,000 cps were reported to die in one minute. This value is plotted in figure 3 and also agrees quite well with the theoretical curves. Shaved mice in the same sound field died after a 3.5-minute exposure (fig. 4). At first glance in the light of the values for *a* measured for the rat, the latter time of 3.5 minutes would appear to be short for a 20,000 cps exposure. However, for skin overlying soft tissues *a* was found by Gierke ('50) to vary inversely with the area measured. Considering the small dimensions of a mouse an absorption coefficient of 0.01 to 0.05 seems possible and would be consistent with the observed 3.5-minute lethal time.

Additional values for furred and shaved mice have been reported by Frings and Senkovitz ('50) for sound fields of 156 db at 6,000 cps and are plotted in figures 3 and 4. Again the lethal times of 60–90 seconds furred and of 4 minutes shaved are consistent with the theory.

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